



## Triclosan removal in wastewater treatment processes

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## **Preface and acknowledgements**

This dissertation is submitted in partial fulfillment of the requirements for obtaining the degree of Doctor of Philosophy (PhD). The dissertation consists of an introduction, a summary of project results with related literature and 8 supporting papers.

This PhD project was carried out at the Department of Biotechnology, Chemistry and Environmental Engineering, Aalborg University during the period December 2008 to November 2012, partly at the xenobiotic group, Department of analytical chemistry, University of Duisburg-Essen and partly at the Department of Environmental Science, Aarhus University. This PhD project has been financially supported by Danish Research Council for Technology and Production (FTP) which is gratefully acknowledged. The ozonation study was supported by German Federal Ministry of Economics and Technology within the agenda for the promotion of industrial cooperative research and development (IGF) based on a decision of the German Bundestag. The sludge reed bed study was supported by ProInno/AIF.

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November 2012

Xijuan Chen

## Table of Contents

<b>Preface and acknowledgements .....</b>	<b>1</b>
<b>Table of Contents .....</b>	<b>3</b>
<b>Abstract.....</b>	<b>4</b>
<b>Resumé (Abstract in Danish) .....</b>	<b>5</b>
<b>List of supporting papers .....</b>	<b>6</b>
<b>Introduction .....</b>	<b>7</b>
1. Triclosan: Usage and characterization .....	7
2. Fate of triclosan in wastewater treatment plants .....	10
3. Occurrence of triclosan in the environment .....	12
4. Risks of triclosan in the environment .....	13
5. Transformation of triclosan in the environment.....	14
6. Objectives of the study .....	17
7. Technologies to remove triclosan after classical activated sludge treatment process .....	18
7.1 Ozonation treatment process (for wastewater) .....	18
7.2 Sludge reed bed treatment process (for sludge).....	20
<b>Project conclusions .....</b>	<b>23</b>
<b>Future perspectives .....</b>	<b>29</b>
<b>References.....</b>	<b>30</b>

## Abstract

Triclosan is an antimicrobial agent which is widely used in household and personal care products. Widespread use of this compound has led to the elevated concentrations of triclosan in wastewater, wastewater treatment plants (WWTPs) and receiving waters. To investigate the occurrence and fate of triclosan in the environment, analytical methods for the quantification of triclosan in wastewater, sludge and deposit were developed.

The fate of triclosan in a wastewater treatment plant (biological degradation, 19%; sorption to sludge, 61%; input into the receiving surface water, 20%) was detected during a field study. From the laboratory-scaled experiment biodegradation products such as 2,4-dichlorophenol, 4-chlorocatechol, triclosan-methyl, monohydroxy-triclosan, dihydroxy-triclosan and triclosan O-sulfate have been identified. In an enrichment culture originated from activated sludge, *Methylobacillus* was identified as one of the active triclosan degraders.

Furthermore, two technologies have been studied for further removal of triclosan in wastewater and sludge. Complete removal of triclosan from wastewater could be achieved by using ozone and more than 95% removal of triclosan from sludge could be found by using reed bed sludge treatment process.

## Resumé (Abstract in Danish)

Triclosan er et bakteriehæmmende middel, som ofte anvendes i husholdningsprodukter og produkter til personlig pleje. Den store anvendelse af stoffet har ført til forhøjede koncentrationer af triclosan i spildevand, rensningsanlæg og vandmiljøet nær udløb fra rensningsanlæg. For at undersøge triclosans forekomst og skæbne i naturen, blev der udviklet analytiske metoder til kvantificering af triclosan i spildevand, slam og sedimenter. Triclosans skæbne i et spildevandsanlæg (biologisk nedbrydning, 19%, absorption i slam, 61%, udledning til det modtagende vandmiljø, 20%) blev målt som en del af et feltstudie. I laboratorie eksperimenter er nedbrydningsprodukter såsom 2,4-dichlorphenol, 4-chlorocatechol, triclosan-methyl, monohydroxy-triclosan, dihydroxy-triclosan og triclosan O-sulfat efter biologisk nedbrydning blevet identificeret. I en beriget kultur, der stammede fra aktivt slam, blev *Methylobacillus* identificeret som en af de, der aktivt nedbryder triclosan.

Desuden er to efterbehandlings-teknologier til at fjerne yderligere triclosan fra spildevand og slam blevet undersøgt. Fuldstændig fjernelse af triclosan fra spildevand kunne opnås ved hjælp af ozon og mere end 95% fjernelse af triclosan fra slammet kan undersøges ved anvendelse af en rodzone slam behandlingsproces.

## List of supporting papers

- Paper 1: **Chen XJ.**, Pauly U., Rehfus S. and Bester K. Personal care compounds in a reed bed sludge treatment system, *Chemosphere* 76 (2009) 1094–1101
- Paper 2: **Chen XJ.**, Pauly U., Rehfus S. and Bester K. Removal of personal care compounds from sewage sludge in reed bed container (lysimeter) studies — Effects of macrophytes, *Science of the Total Environment* 407 (2009) 5743–5749
- Paper 3: **Chen XJ.** and Bester K. Determination of organic micro-pollutants such as personal care products, plasticizer and flame retardants in sludge, *Anal Bioanal Chem* (2009) 395:1877–1884
- Paper 4: Bester K., **Chen XJ.**, Pauly U. and Rehfus S. Abbau von organischen Schadstoffen bei der Klärschlammbehandlung in Pflanzenbeeten, *Korrespondenz Abwasser, Abfall* 58 (2011) 1050-1157
- Paper 5: **Chen XJ.**, Nielsen JL., Furgal K., Liu YL., Lolas IB. and Bester K. Elimination of triclosan and formation of methyl-triclosan in activated sludge under aerobic conditions, *Chemosphere* 84 (2011) 452–456
- Paper 6: **Chen XJ.**, Richard J., Dopp E., Türk J., Liu YL. and Bester K. Ozonisation products of triclosan in advanced wastewater treatment, *Water Research* 46 (2012) 2247-2256
- Paper 7: Lolas IB., **Chen XJ.**, Bester K. and Nielsen JL. Identification of triclosan degrading bacteria using stable isotope probing and microautoradiography. *Microbiology* 158 (2012) 2805-2814
- Paper 8: **Chen XJ.**, Wimmer R., Ternes T., Schlüsener M., Nielsen JL. and Bester K. Biodegradation of triclosan and Formation metabolites in activated sludge under aerobic conditions. Manuscript in preparation

## Introduction

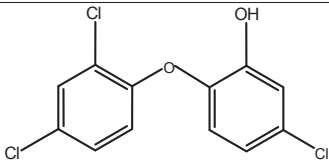
### 1. Triclosan: Usage and characterization

Triclosan (2,4,4'-trichloro-2'-hydroxydiphenylether) is a synthetic, lipid-soluble, broad spectrum antimicrobial agent which was first introduced in the health care industry in 1972 and since 1985 as a compound added to toothpaste in Europe (Joens et al., 2000). It is also referred as Irgasan, DP300, FAT 80'023, CH 3565, and GP 41-353 in a number of toxicology studies. It is a chlorinated organic compound with functional groups representative of both ethers and phenols (Table 1). At normal room temperature, triclosan appears as a white to off-white crystalline powdered solid with a slight aromatic odor and is slightly soluble in water but readily soluble in most organic solvents due to its physicochemical properties which are shown in Table 1.

Triclosan has a specific mechanism to inhibit bacteria activity, i.e. by inhibiting the enoyl-acyl carrier reductase enzyme to block the lipid synthesis, which is necessary for cell reproduction, and therefore prevents the bacteria from synthesizing new fatty acids and thereby stop building cell membranes as well as reproducing (McMurry et al., 1998; Heath et al., 1999).



*Table 1. Physico-chemical parameters of triclosan (MS Search v2.0; EPI Suit 4.0).*

Molecular structure	
Chemical name	2,4,4'-trichloro, 2'-hydroxy-diphenylether
Chemical abstracts service registry (CAS) number	3380-34-5
Synonyms	5-chloro-2-(2,4-dichlorophenoxy)phenol
Trade names	Irgasan; CH 3565, Irgasan CH 3565, Irgasan DP300, Ster-Zac, Tinosan AM110 Antimicrobial, Invasan DP 300R, Invasan DP 300 TEX, Irgaguard_RB 1000, VIV-20, Irgacare MP, Lexol 300, Cloxifenolum, Aquasept, Gamophen, Vinyzene DP 7000, Vinyzene SB- 30, Sanitized Brand, Microbanish R, Vikol THP, Ultra-Fresh, Microban Additive "B", and AerisGuard.
Molecular formula	$C_{12}H_7Cl_3O_2$
Molecular weight	290 g/mol
Vapour pressure	0.00062 Pa
Water solubility	4621 $\mu\text{g/L}$
Boiling point	373.62 $^{\circ}\text{C}$
log Kow	4.2-4.76
log Koc	4.265
pKa	7.9

It is known that triclosan in lower concentrations is recognized as bacteriostatic (Champlin et al., 2005), while it becomes bactericidal in higher concentrations (Suller and Russell, 2000; Russell, 2004). According to the results from Jones et al. (2000),

personal care products which contain 1% of triclosan offers the ideal balance of antimicrobialeffectiveness and mildness for use in high-risk, high-frequency handwashing health care settings. It has shown to be highly active against some microorganisms, such as *Staphylococcus*, *Streptococcus* and *Mycobacterium* (Levy et al., 1999), as well as some fungi and protozoa (i.e. *Plazmodium falciparium*, *Toxoplasma gonidii*) (Yazdankhah et al., 2006). Effectiveness of triclosan is also based on the fact that it can stay on the skin for 12 hours after applied and continues killing bacteria (Glaser, 2004).

In 1965, triclosan was produced by the specialty chemical company “Ciba” in Switzerland (Levy et al., 1999) and then was introduced into personal care products in the USA as an ingredient in deodorants and hand soaps. Due to the bactericidal property and the chemical stability, triclosan became popular as an antimicrobial substance in a wide range of applications and the demand of it has been growing rapidly in the last 40 years (Fiss et al., 2007).

Nowadays, triclosan is used in 140 different types of consumer products including liquid hand soap, shower gels, hand lotions, toothpaste, mouthwashes, deodorants, as well as in the treatment of textiles and in plastic manufacture such as towels, mattresses, toothbrushes, phones, kitchenware and plastic food containers, shoes, clothing and children’s toys (Levy et al., 1999; Fiss et al., 2007). Triclosan has also been used in hospitals and medical products to control bacteria and the spread of disease (Bhargava et al., 1996; Singer et al., 2002).

In the European Union (EU), about 85% of the total amount of triclosan is used in personal care products, compared to 5% in textiles and 10% in plastics and food

contact materials (SCCP/1192/08, 2009). The quantity used within the EU reached approximately 450 tons (as 100% active) in the year 2006 (SCCP/1192/08, 2009).

## **2. Fate of triclosan in wastewater treatment plants**

Widespread use of triclosan provides a number of pathways to enter the wastewater and finally reach the wastewater treatment plants (WWTPs). Triclosan has been detected in the influent of WWTPs in concentrations from ng/L to µg/L (Table 2). In WWTPs using activated sludge as secondary treatment process, about 42-99% removal was detected. The ways of elimination of triclosan in WWTPs include mineralization, transformation by oxidation or reduction and sorption to sludge (Latch, 2003; Bester, 2003, 2005; Yu et al., 2006). In fact, both biodegradation and sorption to activated sludge may explain the removal fate of triclosan in WWTPs.

Although triclosan is a bactericidal compound to many bacteria or fungi, some microorganisms are resistant to triclosan and even able to use it as a sole carbon source. In a continuous activated sludge system study in which the influent level of triclosan was increased from 40 µg/L to 2000 µg/L, removal of triclosan exceeded 98.5% (Federle et al., 2002). Similarly, Stasinakis et al. (2007) have reported that more than 90% removal of triclosan was detected in two continuous-flow activated sludge systems with received triclosan concentration in 500-2000 µg/L. Increasing levels of triclosan concentration had no major adverse on wastewater treatment process, including chemical oxygen demand, biological oxygen demand and ammonia removal (Federle et al., 2002).

*Table 2. Fate of triclosan in different WWTPs.*

Country	Inflow concentration (ng/L)	Effluent concentration (ng/L)	Sludge (ng/g DW)	Removal rate	Literature/Source
USA	670-5115	40-1117		79-97%	Thompson et al., 2005
	4700±1600	70±60	30000±11000	98±1%	Heidler and Halden, 2007
	3800-16600	200-2700	500-15600	96%	McAvoy et al., 2002
	18850	1036	515	95%	Kumar et al., 2010
	13703	180	566	99%	Kumar et al., 2010
	86161	5370	1611	94%	Kumar et al., 2010
	32639	274	795	99%	Kumar et al., 2010
	453-4530	36-212	-	92-97%	Buth et al., 2011
China	142±16.5	22.5±1.4	-	84%	Wu et al., 2007
Germany	1200±80	51±8	1200±130	95-96%	Bester, 2003
	7300±1500	300±100	-	>95%	Bester, 2005
	4800±550	620±1500	-	87%	Bester, 2005
Japan	2680	262	19496	90%	Nakada et al., 2010
	11890	269	-	98%	Nakada et al., 2010
Switzerland	600-1300	11-98	-	94%	Singer et al., 2002
	500-1300	70-650	-	44-92%	Lindström et al., 2002
Australia	586-805	23-434	90-16790	72-93%	Ying and Kookana, 2007

In WWTPs about 30-50% of the triclosan could be recovered in sludge as triclosan is relatively lipophilic with log  $K_{ow}$  of 4.2-4.8 and log  $K_{oc}$  of 4.3 (Bester, 2003, 2005; Coogan et al., 2007; Heidler and Halden, 2007; Kumar et al., 2010; Nakada et al., 2010).

On the other hand, this means that most removal occurs due to biodegradation processes (Heidler and Halden, 2007; Kumar et al., 2010; Nakada et al., 2010).

Due to the incomplete removal triclosan has also been found in the effluent of WWTPs in concentrations of 11-1117 ng/L, whereas typical concentrations of triclosan in sludge were 0.5-30 ng/g dry weight (DW) (Table 2).

### **3. Occurrence of triclosan in the environment**

The widespread use and incomplete removal of triclosan in WWTPs provides a number of pathways to enter the environment. Previous reports described triclosan as one of the most commonly encountered substances in solid and water environmental compartments (Lindström et al., 2002; Singer et al., 2002; Wilson et al., 2003). The chemical properties of triclosan indicate that it may also bioaccumulate and persist in the environment, and laboratory tests have shown it to be toxic to aquatic species, with algae being one of the most sensitive species (*Chlamydomonas*, *Scenedesmus*, *Navicula*, *Synedra*) (Wilson et al., 2003).

Lindström et al. (2002) has detected that the concentrations for triclosan in lakes and a river in Switzerland were up to 70 ng/L. Whereas the concentrations of triclosan in the river Ruhr in Germany was ranged from <3 to 10 ng/L (Bester, 2005). Andresen et al. (2007) has reported that the concentration of triclosan were around 20 ng/L in Hamilton Harbor and approximately 1 ng/L in Lake Ontario. Wilson et al. (2009) have found an average water column concentration of  $3 \pm 2$  ng/L in the lower Hudson River Estuary. Urban wastewater discharge and industrial activities are identified as the main source

for triclosan as high values up to 11-98 ng/L were found in the rivers receiving WWTPs effluents (Bester, 2005).

Triclosan can partition to sewage sludge during wastewater treatment and subsequently transfer to soil when applied to land. High concentrations of triclosan were found in a field receiving biosolid in land application (Langdon et al., 2012). Furthermore, triclosan has also been detected at concentrations of 5-27 ng/g in marine sediments of Barker Inlet, South Australia (Fernandes et al., 2011).

In addition, several studies report that triclosan have been detected with 25-2444 ng/g in the indoor air, which is supposed to be associated with volatilization of liquid personal care products and diffusion from textiles and other materials treated with triclosan as bactericide (Canosa et al., 2007; Fan et al., 2010).

#### **4. Risks of triclosan in the environment**

Considering triclosan has been detected in environment compartments discussed in last subchapter, there is a potential risk of adverse effects in aquatic, terrestrial environment and humans.

Ricart et al. (2010) has reported that triclosan affect both bacterial and algal communities, indicating that the potential environment risk of triclosan is high especially in rivers where water scarcity results in low dilution. Delorenzo et al. (2007) has suggested that triclosan shows low toxicity risk but high potential for chronic and sublethal effects to estuarine organisms. Lin et al. (2012) has reported that triclosan may have potential biochemical and genetic toxicity toward earthworms (*Eisenia fetida*). Considering the PNEC (Predicted No-Effect Concentration, 7ng/L) and NOEC (No Observed Effect Concentration, 700 ng/L) values of triclosan (Thompson et al., 2005),

the risk should be more related to chronic effect (due to the bioaccumulation) than acute impact.

In soils, triclosan has been shown to increase dehydrogenase activity (Ying et al., 2007), and to affect microbial respiration rates (Waller and Kookana, 2009; Butler et al., 2011). The study of Butler et al. (2011) suggests that triclosan inhibits soil respiration but a subsequent acclimation of the microbial community occurs.

Due to the widespread use of triclosan, there is potential for humans to receive exposure, and in fact, triclosan has been detected in human milk (Adolfsson-Eric et al., 2002; Toms et al., 2011), urine (Sandborgh-Englund et al., 2006; Queckenberg et al., 2010) and plasma (Hovander et al., 2002; Sandborgh-Englund et al., 2006), indicating that the human body can be influenced by an individual use of triclosan containing products. It is known that triclosan in lower concentrations may disrupt thyroid function and therefore be recognized as bacteriostatic (Champlin et al. 2005). Guo et al. (2012) has reported that triclosan inhibits adipocyte differentiation of human mesenchyme stem cells under concentrations that are not cytotoxic and in the range (0.1-8.1 µg/L, Sandborgh-Englund et al., 2006) observed in human blood. Additionally, triclosan has also been linked to a range of health effects, such as skin irritation, allergy susceptibility, liver and inhalation toxicity (Coogan et al., 2007).

## **5. Transformation of triclosan in the environment**

Triclosan have triggered an increasing concern for several decades all over the world. It is toxic, last for a long time in the environment, and may travel long distances far from their sources of usage, release and emission. Under the aerobic condition normally present in the sub-surface layers of water, triclosan is expected to undergo primarily

photodegradation and biodegradation (Tixier et al., 2002, Bester, 2005). Triclosan can give derivatives which can be more persistent and more toxic than the parent compound. Furthermore, triclosan and its transformation products can accumulate in fatty tissues of living organism leading to undesirable effect to ecosystem and the human body.

Photodegradation is the dominant degradation pathway for triclosan in surface waters. Tixier et al. (2002) found that direct phototransformation accounted for 80% of the observed total elimination of triclosan from the lake within two months. Degradation products such as chlorophenol, dichlorophenol and dioxin derivatives have been identified (Tixier et al., 2002; Ferrer et al., 2004; Aranami and Readman, 2007; Wong-Wah-Chung et al., 2007; Chen et al., 2008; Son et al., 2009). The degradation products were formed also under environmental conditions in wastewater matrices, thus suggesting their presence in real wastewater treatment process and environmental aquatic systems (Ferrer et al., 2004). Half-life of triclosan photodegradation in the air is about 1 day (Halden and Paull, 2005).

Biodegradation of triclosan has also been described by several investigators. Its half-life is calculated as 11 days in surface water (Bester, 2005), 18-107 days in sludge amended soil (Lozano et al., 2010; Ying et al., 2007; Wu et al., 2009; Waria et al., 2011), 18-78 days in the silty clay loam (Waria et al., 2011; Kookana et al., 2011) and 421 days in the fine sand (Waria et al., 2011) depending on the initial concentration and the environmental factors (Stasinakis et al., 2007). Under these conditions, triclosan-methyl has been reported as the most often occurring transformation product, which presents a greater potential for bioaccumulation than triclosan due to its log  $K_{ow}$  5.0 (Balmer et al., 2004; Waria et al., 2011).



Triclosan-methyl was detected in various aquatic environments such as WWTPs, lakes and rivers (McAvoy et al. 2002; Lindström et al. 2002). Although the concentrations of triclosan-methyl were very low in surface waters with less than 1 ng/L (Balmer et al. 2004), it is bio-accumulating to a high extent, and causes currently the dominating peak when analyzing lipophilic pollutants in fish from urban waters. The concentrations of triclosan-methyl were up to 35 ng/g (wet weight) and up to 365 ng/g on a lipid basis in the fish collected in Switzerland (Balmer et al., 2004). Additionally, occurrence of triclosan-methyl was detected at concentrations <11 ng/g in marine sediments of Barket Inlet, South Australia, which was linked to both wastewater discharges and biological methylation of triclosan (Fernandes et al., 2011).

Biodegradation products such as hydroxycompounds (monohydroxy-triclosan and dihydroxy-triclosan) and the ether bond cleavage products (4-chlorophenol and 2,4-dichlorophenol) have been reported by Kim et al., (2010) and Wu et al. (2010). Veetil et al. (2012) have found that triclosan could be biodegraded under aerobic, anaerobic and anoxic conditions and phenol, catechol and 2,4-dichlorophenol were among the products.

Sonochemical degradation of triclosan in urban runoff and wastewater influent has been investigated by Sanchez-Prado et al. (2008) and suggesting a fast and complete conversion of triclosan without accompanied toxic by-products. Levy et al. (1999) found that under exposure to ozone the antibacterial activity of triclosan is derived primarily from its phenol ring, via van der Waals and hydrogen-bonding interactions with the bacterial enoyl-acyl carrier reductase enzyme.

Furthermore, there are reports that suggest incineration of textile products containing triclosan may result in the formation of dioxin-like substances (NICNAS, 2009).

## **6. Objectives of the study**

Considering there is a severe lack of knowledge on the fate of triclosan in the environment, i.e. 1) by which process and in which part of the treatment plants is triclosan removed and 2) by which process is triclosan-methyl generated, 3) to what compounds is triclosan biodegraded and 4) are there some treatment technologies which can prevent the emission of triclosan to the environment. The primary goal for this project was to address these issues.

- 1) The fate of triclosan in WWTPs was investigated through establishing a complete mass balance of triclosan in single process step analysis in a selected wastewater treatment plant.
- 2) The formation as well as the mass balance of triclosan-methyl was measured in the same wastewater treatment plant.
- 3) The biodegradation of triclosan was analysed through laboratory-scale experiments by using activated sludge bioreactors under aerobic, anaerobic and anoxic conditions.
- 4) Two treatment technologies were involved in this study.

The incomplete removal of triclosan in WWTPs leading to undesirable discharges of triclosan residuals to surface water. Thus experiment on ozonisation of triclosan was carried out to determine the removal rate and transformation products, as well as to assessing the potential of ozonisation as a post-treatment technology in elimination of triclosan in full-scale treatment plant.

Moreover, due to the high partition rate of triclosan to sewage sludge during wastewater treatment and high concentrations of triclosan in the field receiving sludge in land application, removal of triclosan in reed bed sludge treatment technology has been investigated. The goal of the study was to investigate how triclosan and other personal care products (PCPs) were removed and how the different conditions such as temperature, oxygen concentration, macrophytes may affect the triclosan removal in sludge reed beds.

## **7. Technologies to remove triclosan after classical activated sludge treatment process**

### **7.1 Ozonation treatment process (for wastewater)**

As conventional wastewater treatment processes are unable to act as a reliable barrier concerning triclosan, it is discussed to introduce additional advanced treatment technologies in the areas where a persistent pollution problem has been recognized or anticipated. Ternes et al. (2003) and Ikehata et al. (2008) have evaluated different technologies including ozonation and advanced oxidation processes, membrane bioreactor, membrane filtration and activated carbon adsorption, suggesting that chemical oxidation using ozone is a highly effective treatment process for a wide spectrum of emerging organic pollutants, including pesticides, pharmaceuticals, personal care products, surfactants, microbial toxins and natural fatty acids.

Ozone ( $O_3$ ) is a very powerful disinfecting and deodorizing gas. The ability of ozone to disinfect polluted water was recognized in 1886 by de Meritens (Laurie and Gilmore, 2008). However, the widespread introduction of ozone in drinking water treatment

started in the 1960s (Landlais et al., 1991). For a long time ozone has been used in removal of bacteria, vira, algae and fungi as well as sulfur, thus also in eliminating taste and odor problems, as well as in oxidizing and mineralizing organic chemicals (Landlais et al., 1991). Nowadays ozonation followed by granular activated carbon filtration is already a standard method for the treatment of raw surface water to produce drinking water.

However, the experience gained in the drinking water area can also be used in applying the method to wastewater treatment. Ozonation has recently emerged as an important technology for the oxidation and destruction of a wide range of organic pollutants in wastewater (Ikehata et al., 2006). It has been proven to be an effective post-treatment technique for pharmaceuticals and personal care products (Carballa et al., 2007; Ikhata et al., 2008; Wert et al., 2009). Figure 1 show an ozone reactor connected to a sand filter installed after the biological treatment step at the Wüeri WWTP in Regensdorf, Switzerland. Substances reacting fast with ozone, i.e. most of the micropollutants were brought to concentrations below the detection limit by an ozone dose of 0.47 g O<sub>3</sub>/g DOC (dissolved organic carbon) (Hollender et al., 2009; Zimmermann et al., 2011).

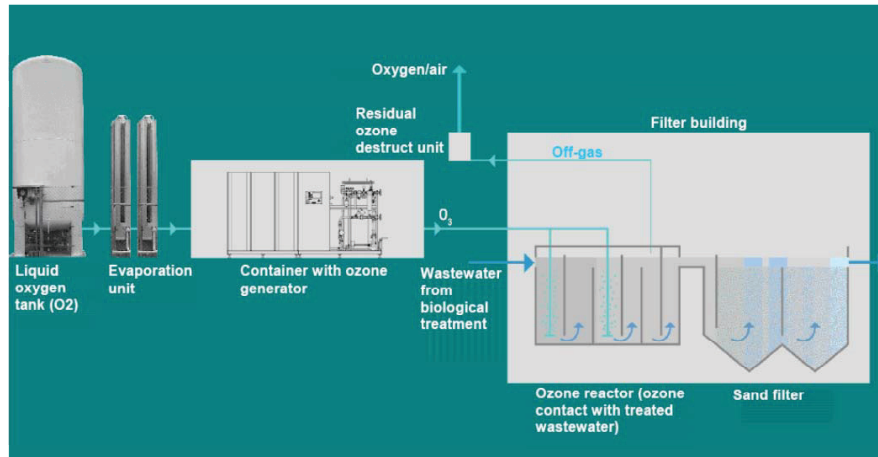


Figure 1. Configuration of the Wüeri WWTP in Regensdorf, Switzerland which has implemented an ozone treatment process. Ozone was produced from liquid oxygen and injected into the existing, but modified flocculation reactor between secondary clarifier and sandfiltration.

Moreover, Suarez et al. (2007) reported that nearly 100% of triclosan depletion was achieved for a 4 mg/L  $O_3$  dose applied to a wastewater containing 7.5 mg/L of DOC, while Wert et al. (2009) reported that >95% triclosan removal was independent of water quality when the  $O_3$  exposure ( $\int O_3 dt$ ) was measurable (0-0.8 mg min/L).

## 7.2 Sludge reed bed treatment process (for sludge)

Sewage sludge (also referred to biosolids) has been used as fertilizer on agricultural land because of its high content of phosphorous and nitrogen (Fytili and Zabaniotou, 2008). This usage of sludge is controversial because of its high content of xenobiotics and heavy metals (Fytili and Zabaniotou, 2008). In 2005 ca. 10 million tons (dry matter) of sludge were produced by sewage treatment plants in Europe, of which approximately 37% of that was used in agriculture (Fytili and Zabaniotou, 2008). Currently sludge in

urban regions is usually stabilised for 10-40 days in anaerobic digesters. However, for rural regions another method of sludge stabilisation has been developed, in which the sludge is treated for about ten years by reed beds to dewater and detoxify the sludge. The reed bed treatment plant is different to conventional dry beds and sludge polders with a new type technology, as

- A) The reed beds are equipped with *Phragmites australis* reeds, which influence the dewatering, and further stabilisation and the sanitizing of the sewage sludge.
- B) The treatment process takes place in dedicated beds, which are separated from the soil and the ground water by polyethylen foil (PE) to prevent the contamination of the soil and groundwater (Pauly et al., 2006).

Reed beds facilities typically consist of several (often 7-10) beds (Figure 2). Each reed bed is lined with a drainage system to enhance the dewatering of the sludge. The leached water is then pumped back into the wastewater treatment plant. The sludge is pumped straight onto the beds throughout the year in pre-determined quantities and at preset intervals. Depending on plant design, the capacity of the beds is exhausted after 6-12 years (Nielsen, 2003). After a resting phase of approximately one year, the individual beds are cleared and are then available again for a fresh loading cycle.

Reed beds for sludge dewatering and mineralization offer an economically attractive alternative to pressure dewatering and centrifugation. Their primary advantages is that they are simple in design and operation, require low manpower (Cooper et al., 2004) and suitable for field or forest application as fertilizer as processed sludge residue complies with agriculture standards (Nielsen and Willoughby, 2007). Nowadays reed beds have been built in many European countries (Haber et al., 1995; Cooper et al.,

2004) and in the USA (Kim and Cardenas, 1990). They have been considered as a low cost and low contamination method considering suspended solids and BOD<sub>5</sub> removal in treating wastewater (Cooper et al., 1999, Wood et al., 2007, Zhao et al., 2008). For the treatment of sludge, true reed beds have been applied in respect of reducing volume, breaking down organic matter and increasing the density of sludge (Nielsen, 2003, 2005, Gustavsson et al., 2007).



*Figure 2. Reed bed facility located in Kalkar-Hönnepel, Germany. It is consisting 7 reed beds covering 20,400 m<sup>2</sup> (outlined in red).*

## Project conclusions

This study was carried out to fill the gap in knowledge concerning the biodegradation and emission of triclosan in WWTPs and further removal of triclosan after the WWTPs.

**Full scale study** (unpublished): In the case study of Aabybro WWTP, 77% of triclosan was removed by the WWTP, which is relatively low due to the low influent concentration. The WWTP was chosen because it has separated denitrification and nitrification process, to detect the removal of triclosan and formation of triclosan-methyl under different conditions. During the treatment process, approximate 23% of the triclosan was emitted to the environment by effluent water; 57% of the triclosan was adsorbed to sludge particles and approximate 20% was biodegraded, in which approximate 5% triclosan transformed into triclosan-methyl as shown in Figure 3. Most of the transformation process (triclosan to triclosan-methyl) occurred in the nitrification process under aerobic conditions. The sorption on sludge contributed significantly to triclosan removal.



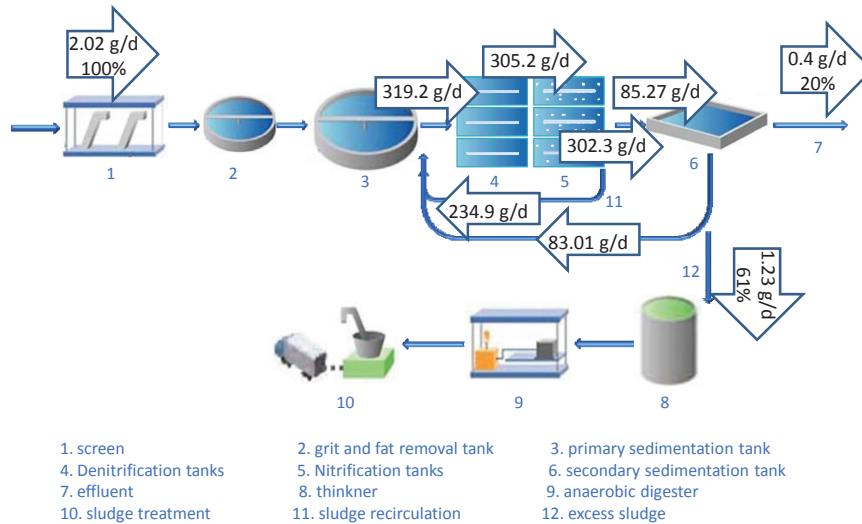


Figure 3. Mass balance of triclosan in Aabybro WWTP.

**Laboratory mechanistic study** (published in paper 5 and 7): In static unfed laboratory-scale activated sludge reactors under aerobic conditions within 7 days, the removal rates (pure biodegradation) of triclosan were relatively lower (75-86%) when the starting concentration was low ( $\leq 0.5 \text{ mg L}^{-1}$ ), and reached higher values (>99%) when the starting concentration was high ( $\geq 1 \text{ mg L}^{-1}$ ), while less removal was observed under anaerobic and anoxic conditions. The tentative half-life of triclosan degradation in sludge reactor under aerobic conditions was estimated to be 54-86 hours. One percent of the triclosan was converted to triclosan-methyl under aerobic conditions, less under anoxic (nitrate reducing) and none under anaerobic conditions. Under aerobic conditions, 10% of triclosan could be recovered in sludge (paper 5) and 7% was transformed into 2,4-dichlorophenol (paper 8). More transformation products such as 4-

chlorocatechol, monohydroxy-triclosan, dihydroxy-triclosan and triclosan O-sulfate have been identified (Figure 4) (paper 8). In an enrichment culture originated from activated sludge, *Methylobacillus* was identified as one of the active triclosan degraders by using DNA based stable isotope probing (DNA-SIP) combined with microautoradiography-fluorescence in situ hybridization (MAR-FISH) (paper 7).

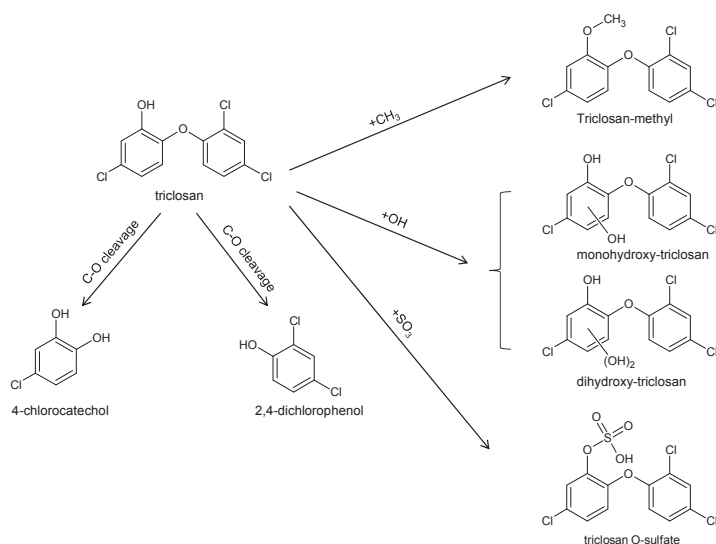


Figure 4. Proposed biodegradation pathway of triclosan.

**Ozonisation study** (published in paper 6): Contamination of surface water and ground water with triclosan is an emerging issue in environment due to the incomplete removal of triclosan in WWTPs. Additional ozonation treatment technology could be used for triclosan removal in drinking water, wastewater and surface water. The treatment process can remove triclosan completely and convert it into the products: 2,4-dichlorophenol, chlorocatechol, monohydroxy-triclosan and dihydroxy-triclosan (paper

6). Increasing the ozone amounts in the reactions leads to decreased concentration of triclosan as well as its oxidation by-products, proving ability to remove triclosan by ozone treatment technology (Figure 5). The transformation product 2,4-dichlorophenol shows lower genotoxic effects than triclosan at the tested concentrations, but this compound is classified to be toxic to aquatic organisms. The other transformation products cannot be assessed up to now.

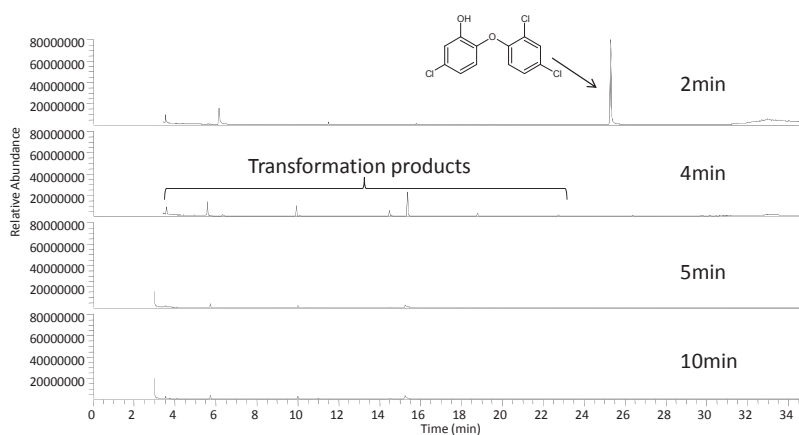


Figure 5. Chromatograms of samples by adding ozone into triclosan solution for 2, 4, 5 and 10 minutes measured by GC/MS.

**Reed bed study** (published in paper 1, 2, 3, 4). The reed bed sludge treatment study showed that the reed bed sludge treatment technology is able to reduce persistent organic pollutant such as bis(2-ethylhexyl) phthalate (DEHP), triclosan, and 1-(2,3,8,8-tetramethyl-1,2,3,4,5,6,7,8-octahydro-naphthalen-2-yl) ethan-1-one (OTNE) significantly. In thirteen months of reed bed sludge treatment process, the concentrations of some

compounds such as OTNE, triclosan, and DEHP in this sludge reed bed treatment were decreased. However, concentrations of other compounds such as polycyclic musk compounds 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-(g)-2-benzopyran (HHCB) and 7-acetyl-1,1,3,4,4,6 hexamethyl-1,2,3,4 tetrahydronaphthalene (AHTN) did not change during this experiment. OTNE and triclosan degraded faster in the bottom layer while DEHP degraded faster in the top layer, which indicates different regimes in the different layers and different degradation processes in the respective layers (Figure 6) (paper 1, 2 and 4).

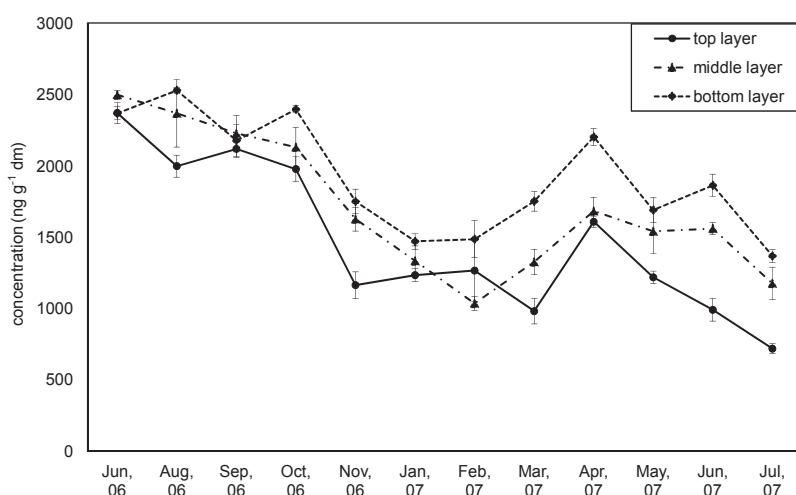


Figure 6. Concentration of triclosan during the experiment period in top layer (0-20 cm from surface), middle layer (20-40 cm from surface) and bottom layer (40-60 cm from surface) of the reed beds. Error bars are from the stated uncertainty from the method development.

Considering half-lives of 300 to 900 days, this sludge reed bed can remove more than 95% of triclosan in its 10 years production cycle. If the sludge is to be used as fertilizer in agriculture the use of reed bed treatments can help considerably to decrease the contamination of sludge. An accounting of material flows in addition showed that only a small fraction (<1%) of the target substances was washed out (leached) with the effluent and the uptake of personal care products into the biomass of the macrophytes can also be neglected.

## **Future perspectives**

In WWTPs using activated sludge as secondary treatment process, most of the removal occurred due to the adsorption and biodegradation in the activated sludge treatment process. In this study biodegradation of triclosan and formation of metabolites have been investigated in laboratory-scaled experiment. However, identification of the metabolites in real WWTPs is strongly needed. Further studies to address bioaccumulation and toxicity of the metabolites will also be required.

Based on the available data, a significant part of triclosan in wastewater is expected to be removed by WWTPs, the residues could be removed by additional ozonation technology. Further research could be focused on assessment of the oxidation capacity of a full-scale ozonation step after the secondary wastewater effluent. Investigation on oxidation of micropollutant as well as formation and removal of byproducts will be necessary.

Regarding the results from the reed bed sludge treatment process, more than 95% of triclosan is expected to be removed after 10 years life cycle. Further research could also be conducted to determine the effectiveness of reed bed technology on other organic micropollutants and the byproducts.

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## Personal care compounds in a reed bed sludge treatment system

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Triclosan

## ABSTRACT

Sewage sludge (also referred to as biosolids) has long been used as fertilizer on agricultural land. The usage of sludge as fertilizer is controversial because of possible high concentration of xenobiotic compounds, heavy metals as well as pathogens. In this study, the fate of the xenobiotic compounds triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol), OTNE (1-(2,3,8,8-tetramethyl-1,2,3,4,5,6,7,8-octahydronaphthalen-2-yl)ethan-1-one), HHCB (1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-(g)-2-benzopyran), HHCB-lactone, AHTN (7-acetyl-1,1,3,4,4,6 hexamethyl-1,2,3,4 tetrahydronaphthalene), and DEHP (bis(2-ethylhexyl)phthalate) in advanced biological treatment of sludge was determined.

During 13 months of field-incubation of the sludge in reed beds, the xenobiotic compounds were analysed. The bactericide triclosan was reduced to 60%, 45%, and 32% of its original concentration in the top, middle, and bottom layer, respectively. The fragrance OTNE was decreased to 42% in the top layer, 53% in the middle layer, and 70% in the bottom layer, respectively. For DEHP a reduction of 70%, 71%, and 40% was observed in the top, middle, and bottom layer, respectively. The polycyclic musk compounds HHCB, AHTN, and the primary metabolite of HHCB, i.e., HHCB-lactone showed no degradation in 13 months during the experimental period in this installation. Tentative half-lives of degradation of triclosan, OTNE and DEHP were estimated to be 315–770 d, 237–630 d, and 289–578 d, respectively.

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## 1. Introduction

Sludge (also referred to as biosolids) has been used as fertilizer on agricultural land because of its high content of phosphorous and nitrogen (Fyttili and Zabaniotou, 2008). This usage of sludge is controversial because of its high content of xenobiotics and heavy metals (Fyttili and Zabaniotou, 2008). In 2005 ca. 10 million tons (dry matter) were produced by sewage treatment plants in Europe. About 37% of that was used in agriculture (Fyttili and Zabaniotou, 2008). Currently sludge in urban regions is usually stabilised for 10–40 d in anaerobic digesters. However, for rural regions another method of sludge stabilisation has been developed, in which the sludge is treated for about 10 years by reed beds to dewater and detoxify the sludge.

The reed bed treatment plant is different to conventional dry beds and sludge polders with a new type technology, as

- (A) The reed beds are equipped with *Phragmites australis* reeds, which influence the dewatering, and further stabilisation and the sanitizing of the sewage sludge.
- (B) The treatment process takes place in dedicated beds, which are separated from the soil and the ground water by polyethylene foil (PE) (Fig. 1) to prevent the contamination of the soil and groundwater (Pauly et al., 2006).

Each reed bed is lined with a drainage system to enhance the dewatering of the sludge. The leached water is then pumped back into the wastewater treatment plant. The sludge is pumped straight onto the beds throughout the year in pre-determined quantities and at preset intervals. Depending on plant design, the capacity of the beds is exhausted after 6–12 years (Nielsen, 2003). After a resting phase of approximately one year, the individual beds are cleared and are then available again for a fresh loading cycle.

A similar technology as reed beds, i.e. soil filters, has been considered as a low cost and low contamination method considering suspended solids and BOD<sub>5</sub> removal in treating wastewater (Cooper et al., 1999; Wood et al., 2007; Zhao et al., 2008). For the treatment of sludge, true reed beds have been applied in respect of reducing volume, breaking down organic matter and increasing the density of sludge (Nielsen, 2003, 2005; Gustavsson et al., 2007), however whether this enhanced biological treatment is suitable for degradation of xenobiotics, was not fully elucidated before undertaking the study presented here. This project was conducted to study whether reed bed treatment of sludge is suited to decrease the loading of xenobiotics to agricultural land with by fertilising with sewage sludge.

## 1.1. Compounds included in this study

Musk fragrances such as HHCB (1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-(g)-2-benzopyran), AHTN (7-acetyl-1,1,3,4,4,6 hexamethyl-1,2,3,4 tetrahydronaphthalene) which is

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E-mail addresses: [kb@bio.aau.dk](mailto:kb@bio.aau.dk), [kai.bester@uni-DUE.de](mailto:kai.bester@uni-DUE.de) (K. Bester).

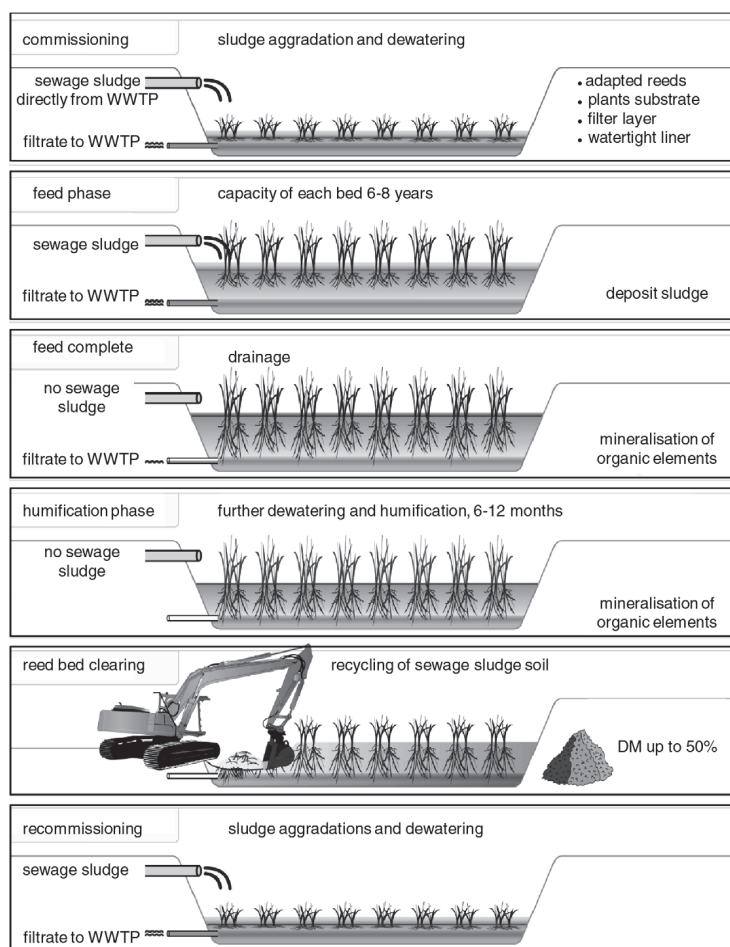


Fig. 1. Sludge treatment process in reed bed treatment plant (Pauly et al., 2006).

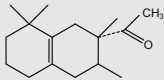
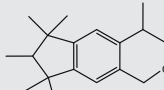
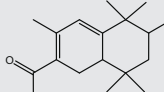
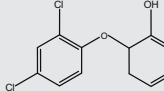
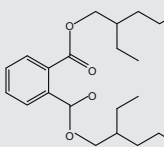
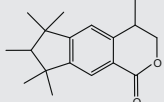
mainly used in domestic purpose as well as OTNE (1-(2,3,8,8-tetramethyl-1,2,3,4,5,6,7,8-octahydro-naphthalen-2-yl)ethan-1-one), triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol), DEHP (bis(2-ethylhexyl)phthalate) have recently been identified as major anthropogenic organic contaminants in sewage sludge (Simovich et al., 2002; Kinney et al., 2006).

Triclosan is currently used as an antimicrobial agent in toothpaste, mouthwash, and in functional clothing such as sport shoes and underwear and as a stabilizing agent in a multitude of detergents and cosmetics (Adolfsson-Erici et al., 2002). Additionally, it is used as an antimicrobial agent in polymeric food cutting boards. Approximately 1500 tonnes are produced annually worldwide, and approximately 350 tonnes of those are applied in Europe (Singer

et al., 2002). Triclosan has a low water solubility and very high potential of bio-accumulation (Coogan et al., 2007). Studies have increasingly linked triclosan to a range of health and environmental effects, skin irritation, allergy susceptibility, and ecological toxicity to the aquatic and terrestrial environment (Coogan et al., 2007). In sludge from North Rhine-Westphalia, triclosan is widespread and the concentration is in the range of more than 2000–8000 ng g<sup>-1</sup> (dry mass) (Bester, 2005a). In Table 1 the structural formula and other details on the compounds are presented.

Polycyclic musk compounds such as HHCB and AHTN are used frequently as fragrances in washing softeners, shampoos, and other consumer products. More than 2000 tonnes are used annually in Europe (Balk and Ford, 1999). The structural formulas of both

**Table 1**  
Compounds of interest.

OTNE (Gautschi et al., 2001; Bester et al., 2008)		Name: 1-(2,3,8,8-tetramethyl-1,2,3,4,5,6,7,8-octahydro-naphthalen-2-yl)ethan-1-one Molecular formula: $C_{16}H_{26}O$ Molecular weight: 234 g mol <sup>-1</sup> CAS: 54464-57-2 Boiling point: 230 °C Vapour pressure: 0.2 Pa Water solubility: 2.68 mg L <sup>-1</sup> log $K_{ow}$ : 5.7 log $K_{oc}$ : 4.64
HHCB (Balk and Ford, 1999)		Name: 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-(g)-2-benzopyran Molecular formula: $C_{18}H_{26}O$ Molecular weight: 258 g mol <sup>-1</sup> CAS: 1222-05-5 Vapour pressure: 0.0682 Pa Water solubility: 1.25 mg L <sup>-1</sup> log $K_{ow}$ : 5.7 log $K_{oc}$ : 4.80
AHTN (Balk and Ford, 1999)		Name: 7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetrahydronaphthalene Molecular formula: $C_{18}H_{26}O$ Molecular weight: 258 g mol <sup>-1</sup> CAS: 1506-02-1 Vapour pressure: 0.0727 Pa Water solubility: 1.75 mg L <sup>-1</sup> log $K_{ow}$ : 5.9 log $K_{oc}$ : 4.86
Triclosan (Bester, 2005a; Ying et al., 2007)		Name: 5-chloro-2-(2,4-dichlorophenoxy)phenol Molecular formula: $C_{12}H_7Cl_3O_2$ Molecular weight: 290 g mol <sup>-1</sup> CAS: 3380-34-5 Vapour pressure: 0.00062 Pa Water solubility: 4.621 mg L <sup>-1</sup> Boiling point: 373.62 °C log $K_{ow}$ : 4.2–4.76 log $K_{oc}$ : 4.265
DEHP (Cheng et al., 2008)		Name: bis(2-ethylhexyl)phthalate Molecular formula: $C_{24}H_{38}O_4$ Molecular weight: 391 g mol <sup>-1</sup> CAS: 117-81-7 Boiling point: 230 °C Vapour pressure: 0.000034 Pa Water solubility: 0.003 mg L <sup>-1</sup> log $K_{ow}$ : 7.5 log $K_{oc}$ : 5.2
HHCB-lactone (Bester, 2005b)		Name: 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta [g]-2-benzopyran-1-one Molecular formula: $C_{18}H_{24}O_2$ Molecular weight: 272 g mol <sup>-1</sup> CAS: 507442-53-7 log $K_{ow}$ : 4.7

compounds are given in Table 1. After application, most of these materials are released to the sewer. Thus, they have been identified in sewage treatment plants (Eschke et al., 1994, 1995) and in the sewage sludge (Reiner and Kannan, 2006). Both of them have very low water solubility and high potential of bio-accumulation, thus they can cause ecological toxicity to the aquatic and terrestrial environment (Brunn and Rimkus, 1997). The musk compounds are not mineralized in sewage treatment processes and sorption is their main elimination path in waste water treatment plants, although transformation to other compounds may occur (Bester, 2005b). Elimination rates of fragrance compounds in 17 different plants in US and Europe were compared by Simonich et al. (2002). Removal rates of 50%–90% were determined for HHCB and AHTN. Concentration of HHCB for  $3100 \pm 240$  ng g<sup>-1</sup> and AHTN for  $1500 \pm 150$  ng g<sup>-1</sup> in digested, dewatered sludge was

determined from one STP in North Rhine-Westphalia (Bester, 2004).

HHCB-lactone is the primary metabolite of HHCB, which is an oxidation product as shown in Table 1. The ratio of HHCB: HHCB-lactone has been used to detect transformation processes of this fragrance. During the sewage treatment process about 10% of HHCB is transformed to HHCB-lactone which has been reported for balance assessment for polycyclic musk fragrances in German treatment plant by Bester (2004). The relation HHCB:HHCB-lactone varies from 3 to 130 in surface waters. This indicates that degradation processes, especially degradation/transformation efficiency, in the respective sewage treatment plants differ considerably (Bester, 2005b). Concentrations of HHCB-lactone from sludge of 20 sewage treatment plants were determined from 30 ng g<sup>-1</sup> to 36,000 ng g<sup>-1</sup> (Bester, 2005b).



OTNE is widely used in consumer products (in Table 1). It has been among the most popular compounds in fragrances in the last few years. It is marketed as Iso E Super, with 2500–3000 tonnes annually being sold (Gautschi et al., 2001). Concentrations of 7000–30,000 ng g<sup>-1</sup> OTNE in dry sludge were determined in sludge from the U.S. (Difrancesco et al., 2004), while European data indicate concentrations of 2000–4000 ng g<sup>-1</sup> (Bester et al., 2008).

DEHP is widely used as plasticizer in PVC construction materials, and also in varnish, paint, and cosmetics products. DEHP is used as a plasticizer because of its stability, fluidity, and low volatility (Giam et al., 1984). This plasticizer is eluted into wastewater by washing and cleaning processes, it is assumed to at least have strong ecotoxic effect to the aquatic organisms (Roh et al., 2007). Because of the relatively high lipophilicity of the compounds, sorption is the main process relevant for elimination in sewage treatment plants. Beauchesne et al. (2008) investigated that sludge can represent significant sources of plasticizers in the environment. Typical concentration of DEHP in sludge was investigated in the range of 10–100 µg L<sup>-1</sup> by Fromme et al. (2002).

According to the parameters shown in Table 1, these compounds have low vapour pressure and low water solubilities. High octanol–water partition coefficient ( $K_{ow}$ ) and soil organic matter–water partitioning coefficient ( $K_{oc}$ ) suggest that these compounds are hydrophobic and sorption is the main process of elimination in sewage treatment plant.

## 2. Materials and methods

AHTN, triclosan, and DEHP were purchased from Ehrenstorfer (Augsburg, Germany) as pure compounds with purity being >99% according to the supplier. OTNE and pure standards of HHCb-lactone as well as HHCb were obtained from International Flavours and Fragrances (IFF, Hilversum, Netherlands).

The internal standard MX D<sub>15</sub> was used to quantify the musks and DEHP as it elutes in the same fraction as these compounds, while TPP D<sub>15</sub> was used in this experiment to quantify triclosan and HHCb-Lactone. While Musk xylene D<sub>15</sub> was obtained from Ehrenstorfer (Augsburg, Germany), TPP D<sub>15</sub> was synthesized from D<sub>6</sub> phenol and phosphorochloride. These internal standards were chosen as they give undisturbed signal, and also not undergo any reaction themselves (Andresen and Bester, 2006).

Ethyl acetate, acetone, and cyclohexane were used in analytical grade (p.a.) quality, while toluene and *n*-hexane were used in residue grade (z.R.) quality. All solvents were purchased from Merck, Darmstadt, Germany.

Samples were taken from the sludge reed bed from the wastewater treatment plant (WWTP) in Meppen, which processes 2,000,000 m<sup>3</sup> wastewater of 52,500 inhabitants annually. About half of the wastewater that is produced is domestic. This WWTP includes primary sedimentation basins, activated sludge treatment basins and a final clarifier before the water is released to the river. This plant produces about 40,000 m<sup>3</sup> excess sludge annually. Until 2003 the sludge was treated by a filter press and then used as fertilizer in agriculture. Since June 2003 the sludge is treated in a reed bed installation consisting of seven separated beds with separated drainage systems (Fig. 1). The reed beds (50 × 20 m each) are equipped by polyethylene foil (PE). To study whether this enhanced biological treatment is suitable for degradation of xenobiotics, one of the reed beds was put out of operation in 2006 and no new sludge was added during the experimental period. The experiment was conducted under ambient conditions: temperature, water content and reed plant density were not changed but as established by nature in this bed. Monitoring of the height of the sludge in this bed proved a stable bed with little alterations 65 cm with 2 cm standard deviation.

From June, 2006 to July, 2007 sludge samples were taken by using a stainless steel tube with a cutting edge for easy core removal. The samples were divided into three sub-samples according to depth. The upper third of the sample is considered to be the top layer, middle third as the middle layer and lower third as the bottom layer. Then 100 g samples were taken from 10 different points of the reed bed and a homogenate for the respective layer was produced.

Two hundred grams of these homogenates were immediately frozen in refrigerating room at -27 °C overnight. Frozen sub-samples of 50 g wet weight were lyophilised at 2 mbar and -46 °C. Duplicates of the lyophilised sludge samples were extracted by means of accelerated solvent extraction (ASE) with ethyl acetate at 90 °C and 150 bar. The resulting extracts were then cleaned up with 1 g silica (SPE) solid-phase extraction cartridges (silica 60 obtained from Merck, Darmstadt, Germany) by elution with ethyl acetate after adding an aliquot of 100 µL internal standard solution (IS) (containing 100 ng D<sub>15</sub> musk xylene and 100 ng TPP D<sub>15</sub>).

These resulting solutions were concentrated to 1 mL by a Büchi multiport concentrator at 80 °C and 70 mbar (Büchi, Essen, Germany). The resulting extracts were injected to a GPC-column (LC-tech, Dorfen, Germany, equipped with Biorad SX-3) ID: 2.5 cm, length 30 cm, flow 5.0 mL min<sup>-1</sup> cyclohexane: ethyl acetate 1:1. The solvent eluting in the first 19:30 min was drained to waste, while the fraction 19.30–30.00 min was collected. Thus, macromolecules were separated as they elute in the first fraction, while sulphur, etc. are separated from the target compounds as they are eluted after the analyte fraction. The samples were finally transferred into toluene. The resulting extracts were finally fractionated on silica using 5% Methyl-*tert*-butylether (MTBE) in *n*-hexane and ethyl acetate successively as eluents. These fractions were condensed and finally analysed by gas chromatography with mass spectrometric detection (GC–MS) equipped with a programmable temperature vapouriser (PTV) injector. The PTV (1 µL injection volume) was operated in PTV splitless mode. The injection temperature of 115 °C was held for 3 s, it was successively ramped with 12 °C s<sup>-1</sup> to 280 °C for the transfer of the analytes. This temperature was held for 1.3 min. The injector was then ramped with 1 °C s<sup>-1</sup> to 300 °C which was held for 7 min as a cleaning phase.

The GC separation was performed with a DB-5MS column (J&W Scientific), L: 15 m; ID: 0.25 mm; film: 0.25 µm and a temperature programme of: 100 °C (hold: 1 min) ramped with 30 °C min<sup>-1</sup> to 130 °C and with 8 °C min<sup>-1</sup> successively to 220 °C. Finally, the baking temperature was reached by ramping the column with 30 °C min<sup>-1</sup> to 280 °C which was held for 7 min.

The detector of the mass spectrometer (DSQ, Thermo Finnigan, Dreieich, Germany) was operated with 1281 V on the secondary electron multiplier and about 40 ms dwell time in selected ion mode (SIM) mode. The transfer line was held at 250 °C, which is sufficient to transfer all compounds from the GC into the MS as the vacuum builds up in the transfer line. The ion source was operated at 230 °C. Helium was used as carrier gas with a flow rate of 1.3 mL min<sup>-1</sup>.

When the rain water passes through the sludge layer, some compounds can be dissolved, which can also lead to the concentration reduction of compounds. Thus, liquid samples were collected as manual grab samples in two litre glass bottles from the drainage water of the drainage canal of the reed bed during the treatment process. Two samples for out-flowing water were taken as duplicates. One litre samples were extracted for 20 min with 20 mL toluene by means of vigorous stirring with a teflonized magnetic stir bar after adding an aliquot of 100 µL internal standard solution. The organic phase was separated from the aqueous one and the residual water was removed from the organic phase by freezing the samples overnight at -20 °C. The resulting extracts were then concentrated with a rotary evaporator at 80 °C and 70 mbar to 1 mL. Resulting extracts were quantified by using GC–MS.

**Table 2**

Quality assurance data including the MS data (analytical and verifier ions) as well as recovery rate standard deviation and limit of quantification of the recovery rate experiments.

Analyte	Analytical ion (amu)	Verifier ion (amu)	Recovery (%)	RSD (%)	LOQ (ng g <sup>-1</sup> )
OTNE	191	219	60	6	30
HHCB	243	258	77	6	10
AHTN	243	258	69	5	10
Triclosan	288	290	133	12	100
HHCB-lactone	257	272	65	10	30
DEHP	279	167	87	21	10

The compounds were detected by means of their mass spectral data and retention times. For quantitative measurements the method was validated, by determining recovery rates, standard deviations, and limits of quantification (see next paragraph) (Bester, 2004, 2007, 2009; Peck, 2006).

The average of the concentrations obtained from the duplicate extractions was used for further data processing. The calibrations were performed as a multi-step internal standard calibration (10–10,000 ng mL<sup>-1</sup>). The recovery rates were assessed by extracting spiked manure/soil 1/1 samples. Six different concentrations (between 20 ng g<sup>-1</sup> and 10,000 ng g<sup>-1</sup>) were dosed, for each concentration two samples were extracted, thus 12 extractions were performed plus extractions for blank determination. Additionally recovery rates were determined by means of standard addition by spiking sludge from this experiment with respective standard concentrations of 5000 ng g<sup>-1</sup>.

#### 2.1. Method quality assurance

The recoveries were 60–133% for the respective compounds, the relative standard deviations varying for the specific compounds from 5 to 21% (for more details see Table 2). The limit of detection was 3–30 ng g<sup>-1</sup> and the limit of quantification was 10–100 ng g<sup>-1</sup>. Limits of quantification (LOQ) were calculated by two means:

- (1) From the analysis of standard solutions, as the lowest concentration which gave signal to noise ratios (s/n) of at least 10 (six replicates in each series).
- (2) As the lowest concentration for the respective substance that was detectable from the recovery studies with the same recovery rate as the higher concentrations. Full data are given in Table 2 (duplicates per concentration).

### 3. Results and discussion

#### 3.1. Water content

During the 13 months field-incubation of the organic compounds in a technical reed bed sludge treatment the water content during time was analysed. The liquid excess sludge (used as feed for this sludge treatment) contained about 99% water. The rainfall in this treatment facility (May 2006–July 2007) was 1130 L m<sup>-2</sup>. The water content of the sludge ranged from 85% to 73% during the experiment period. The lowest water content in the top layer, 76%, was found in September 2006, because of the low amount of rainfall and high temperature (and enhanced transpiration by the reed plants) at that time.

#### 3.2. Personal care compounds

The xenobiotic compounds triclosan, HHCB, AHTN, HHCB-lactone, OTNE, and DEHP were identified by their retention times and mass spectral data in sludge samples (Table 2).

The concentration of triclosan (Fig. 2) in the beginning of the experiment was measured as 1400, 1900, and 2000 ng g<sup>-1</sup> (dry

mass) in the top, middle, and bottom layer, comparable results were obtained by Bester (2005a) in sewage sludge samples from 20 WWTPs in Germany with triclosan concentration ranging from 400 to 8800 ng g<sup>-1</sup>. After 13 months triclosan was reduced to less than 60% and the concentration was 800, 900 and 600 ng g<sup>-1</sup> (dry mass) in the top, middle, and bottom layer, respectively (Fig. 2). Considering a standard deviation of 12% from the method validation this change is significant.

The concentrations of the polycyclic musk compounds HHCB, AHTN, and the primary metabolite of HHCB, i.e. HHCB-lactone showed no reduction in 13 months during the experimental period. The concentration varied from 8000 to 12,000 ng g<sup>-1</sup> (dry mass) for HHCB, from 1500 to 2300 ng g<sup>-1</sup> (dry mass) for AHTN and from 1400 to 2100 ng g<sup>-1</sup> (dry mass) for HHCB-lactone. These are corresponding to the results obtained by Reiner and Kannan (2006) who found concentrations ranging from 7230 to 108,000 ng g<sup>-1</sup> (dry mass) for HHCB, 809 to 16,800 ng g<sup>-1</sup> (dry mass) for AHTN and 3160 to 22,000 ng g<sup>-1</sup> (dry mass) for HHCB-lactone. Nevertheless, a few studies indicated that polycyclic musks can be degraded in sludge-amended soils. Litz et al. (2007) investigated aerobic dissipation of HHCB and AHTN in soil/sewage sludge mixtures is very slow with half-lives of 10–17 months for HHCB and 2–24 years for AHTN. Similarly, Difrancesco et al. (2004) also found a particularly slow dissipation for HHCB and AHTN in sludge-amended soils. Information on degradation of HHCB-lactone is rare, only some mass balance measurement have been carried out that indicate HHCB-lactone is developed during HHCB transformation process (Bester, 2004; Berset et al., 2004; Reiner and Kannan, 2006).

Fig. 2 shows the OTNE concentration as a function of time. The highest amount was determined in the beginning of the project. The measured concentrations were 2500 ng g<sup>-1</sup>, 2500 ng g<sup>-1</sup>, and 2400 ng g<sup>-1</sup> (dry mass) in the top, middle, and bottom layer, this is somewhat lower than found for sludges from the US by Difrancesco et al. (2004). After 13 months OTNE was reduced by 42% in the top layer, 53% in the middle layer, and 70% in the bottom layer, respectively.

Similar to OTNE, the highest concentration of DEHP was detected in the beginning of the project. The respective concentrations were 11,500, 10,500, and 7200 ng g<sup>-1</sup> (dry mass) in the top, middle, and bottom layer. Comparable results were obtained by Beauchesne et al. (2008) ranging from 15,000 ng g<sup>-1</sup> to 346,000 ng g<sup>-1</sup> in sewage sludge in Canada. After 13 months DEHP was reduced by 70%, 71%, and 40% in the top, middle, and bottom layer, respectively.

The processes that contributed to the dissipation of the studied compounds in sludge may include volatilization, plants uptake, leaching, and biological transformation (aerobic and anaerobic). Considering their generally low volatility (Table 1), the tendency of these compounds to volatilize is low. Therefore it is expected that only a small fraction of these compounds was volatilized into the atmosphere, where they can photolyze (Aschmann et al., 2001; Difrancesco et al., 2004; Chen et al., 2008). To quantify the uptake of xenobiotic compounds by plants, reed samples were analysed by using the same procedure as sludge. In these samples none of the compounds were detected, except small amounts of DEHP (13,000 ± 2000 ng g<sup>-1</sup>). As less than 1 kg reeds were growing in

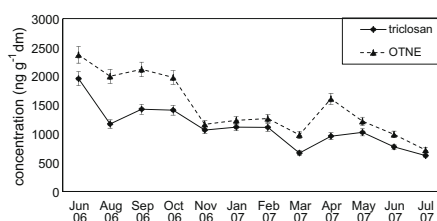


Fig. 2. Triclosan and OTNE concentration from the bottom layer (40–60 cm from surface) as a function of time. Error bars are from the stated uncertainty from the method development.

1 m<sup>2</sup> sludge reed bed, it can be assumed that less than 0.01% of DEHP was ingested by reeds. This is in agreement with the results of Litz et al. (2007) who studied uptake of HHCb and AHTN by lettuce and carrots and found HHCb and AHTN were taken up only by the carrot roots to some small extent. Phyto-remediation (considering only plant uptake) is thus not relevant for this system.

### 3.3. Mass balance studies

The amount of compounds in leachate can be calculated based on the concentration of effluent and amount of rainfall (water flow through the system). The concentration of xenobiotics in the effluent from this reed bed in November 2006 is shown in Table 3. The rainfall during the experimental period (from June 2006 to July 2007) was 1130 mm (Table 3) (1 mm = 1 L m<sup>-2</sup>). Table 3 shows mass fraction of compounds which were leached by drainage water in comparison to the mass fraction in sludge in 1 m<sup>2</sup> reed bed. Since 0.010–0.048% of the mass fraction of the xenobiotics contained in the sludge is leached by drainage water during the experimental period, it seems that biological transformation was the main dissipation mechanism for these compounds.

### 3.4. Kinetic analysis of dissipation data

Biological degradation of organic compounds at low concentrations usually follows first-order kinetics, thus an elimination rate constant ( $k$ ) for sludge removal in reed beds can be calculated from the concentrations from a  $\log c \times c_0^{-1}$  plot (Fig. 3) using Eq. (1). For the triclosan degradation process the respective  $k$  values are 0.0009, 0.0021 and 0.0022 in the top, middle, and bottom layer.

$$K = \frac{\ln(\frac{c_0}{c})}{t} \quad (1)$$

With Eq. (2), the half-life can be assessed:

$$T_{1/2} = \frac{\ln(\frac{c_0}{\frac{c_0}{2}})}{k} = \frac{\ln 2}{k} \quad (2)$$

Table 3

Mass fraction (M1) of compounds dissolved in rain water passing through 1 m<sup>2</sup> reed bed in comparison to the mass fraction in sludge (M2) in 1 m<sup>2</sup> reed bed. As well as relative amount of substance washed of the reed bed with the drainage water (M1/M2). Sludge density is 0.8 ton m<sup>-3</sup>.

Compounds	C1 Drainage water (ng L <sup>-1</sup> )	Rainfall (L m <sup>-2</sup> )	M1 Drainage water (mg)	C2 Sludge (ng g <sup>-1</sup> )	Depth of the reed bed (m)	M2 Sludge (mg)	M1/M2 (%)
OTNE	130	1130	0.147	2370	0.6	1140	0.013
HHCb	310	1130	0.35	9150	0.6	4390	0.0080
AHTN	50	1130	0.057	1220	0.6	586	0.010
Triclosan	270	1130	0.305	1330	0.6	638	0.048
DEHP	170	1130	0.192	11,600	0.6	5570	0.0034

C1: Concentration in the drainage water (ng L<sup>-1</sup>).

C2: Concentration in the sludge (ng g<sup>-1</sup>) (start of experiment).

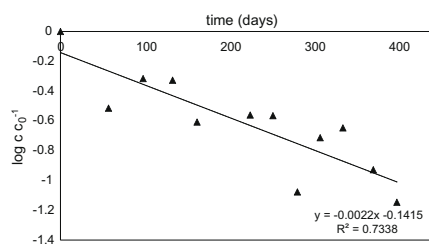


Fig. 3. Kinetics of triclosan degradation in log form in the bottom layer of a sludge reed bed.

Table 4

Tentative half-lives of the compounds during the experimental period in a sludge reed bed. The  $R^2$  refers to the regression line in the log plots to gain the half-life values.

Compounds	Tentative half-life
OTNE	Top layer (0–20 cm) 630 d ( $R^2 = 0.7361$ ) Middle layer (20–40 cm) 239 d ( $R^2 = 0.6047$ ) Bottom layer (40–60 cm) 277 d ( $R^2 = 0.4716$ )
HHCb	Top layer (0–20 cm) ∞ Middle layer (20–40 cm) ∞ Bottom layer (40–60 cm) 770 d ( $R^2 = 0.4858$ )
AHTN	Top layer (0–20 cm) ∞ Middle layer (20–40 cm) ∞ Bottom layer (40–60 cm) ∞
DEHP	Top layer (0–20 cm) 315 d ( $R^2 = 0.6562$ ) Middle layer (20–40 cm) 289 d ( $R^2 = 0.6554$ ) Bottom layer (40–60 cm) 578 d ( $R^2 = 0.4117$ )
Triclosan	Top layer (0–20 cm) 770 d ( $R^2 = 0.4822$ ) Middle layer (20–40 cm) 330 d ( $R^2 = 0.7588$ ) Bottom layer (40–60 cm) 315 d ( $R^2 = 0.7338$ )
HHCb-lactone	Top layer (0–20 cm) ∞ Middle layer (20–40 cm) ∞ Bottom layer (40–60 cm) ∞

∞ means half-lives larger than 3 years.

Tentative half-lives for triclosan can be calculated as 770, 330, and 315 d in the top, middle, and bottom layer, respectively (Table 4). This is corresponding to the results which were gained by Ying et al. (2007) by spiking triclosan into loamy soil with a concentration of 1 mg kg<sup>-1</sup> (i.e. 1000 ng g<sup>-1</sup>), 18 d half-life was calculated under aerobic conditions within this 70 d experiment.

Tentative half-lives of OTNE degradation were calculated as 630, 239, 277 d in the top, middle, and bottom layer, respectively (Table 4), which indicate OTNE degraded faster in the middle and bottom layer than in the top layer. Comparable half-lives were observed by Di Francesco et al. in 2004 with OTNE dissipation half-lives of 30–100 d in sludge-amended soils.

DEHP was eliminated with half-lives as 315, 289, and 578 d in the top, middle, and bottom layer, respectively. These can be compared with results obtained by Madsen et al. (1999) who found that more than 41% of DEHP in a sludge-amended soil was still not mineralization after 1 year incubation and in this study a half-life for DEHP in soils with sludge aggregates was estimated to be higher than 3 years.

### 3.5. Comparison of layering

Triclosan and OTNE degraded very similar concerning the layers of sludge, i.e., faster in bottom layer than in the top layer.

This might be influenced by different age, compactness or oxygen supply in the different layers. The oxygen regime in the different layers that can be quite diverse, as reed is known to pump oxygen from the leaves to the rhizome into the surrounding medium (sludge) (Armstrong et al., 2000). This can be accounted for  $29\text{--}60\text{ ng m}^{-2}\text{ min}^{-1}$  (Armstrong et al., 2000). However the surrounding sludge can consume the oxygen rapidly especially if it is partially aerobically stabilized sludge as in this experiment. During the experiment, the reed bed was monitored in intervals for aerobic and anaerobic areas. The reed bed was usually patchy, thus aerobic areas occurred as well as anaerobic ones. Additionally air could have entered from the drainage basin. The main result at this moment is, there is indeed an effect of the different layers future research will show what might be the reason for that.

By the way of contrast DEHP degraded faster in the top layer, which suggesting the highest reduction of DEHP was achieved at the highest temperature (Cheng et al., 2008). Possibly the degradation of the different compounds is preferred at different oxygen levels (aerobic and anaerobic processes).

### 4. Conclusions

In the 13 months of this experiment, the concentrations of some compounds such as OTNE, triclosan, and DEHP in this sludge reed bed treatment were decreased. However, the concentrations of other compounds such as polycyclic musk compounds HHCB, AHTN, and HHCB-lactone did not change during this experiment. OTNE and triclosan degraded faster in the bottom layer while DEHP degraded faster in the top layer, which is indicating different regimes in the different layers and different degradation processes in the respective layers.

Considering half-lives of 300–900 d, this sludge reed bed can eliminate considerable amounts of some of the pollutants in its 10 years production cycle. If the sludge is to be used as fertilizer in agriculture the use of reed bed treatments can help considerably to decrease the contamination of sludge.

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### Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chemosphere.2009.04.023.

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## **Research paper 2:**

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## Removal of personal care compounds from sewage sludge in reed bed container (lysimeter) studies – Effects of macrophytes

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### ABSTRACT

Sludge reed beds have been used for dewatering (draining and evapotranspiration) and mineralisation of sludge in Europe since 1988. Although reed beds are considered as a low cost and low contamination method in reducing volume, breaking down organic matter and increasing the density of sludge, it is not yet clear whether this enhanced biological treatment is suitable for degradation of organic micro-pollutants such as personal care products. Within this project the effect of biological sludge treatment in a reed bed on reducing the concentrations of the fragrances HHCB, AHTN, OTNE was studied as on the bactericide Triclosan. Additionally, the capacity of different macrophytes species to affect the treatment process was examined. Three different macrophyte species were compared: bulrush (*Typha latifolia*), reed (*Phragmites australis*) and reed canary grass (*Phalaris arundinacea*). They were planted into containers (lysimeters) with a size of 1 m × 1 m × 1 m which were filled with 20 cm gravel at the bottom and 50 cm sludge on top, into which the macrophytes were planted. During the twelve months experiment reduction of 20–30% for HHCB and AHTN, 70% for Triclosan and 70% for OTNE were determined under environmental conditions. The reduction is most likely due to degradation, since volatilization, uptake into plants and leaching are insignificant. No difference between the containers with different macrophyte species or the unplanted containers was observed. Considering the usual operation time of 10 years for reed beds, an assessment was made for the whole life time.

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### 1. Introduction

Reed beds have been used for dewatering and mineralisation of sludge in Europe since 1988 (Nielsen et al., 1992). In comparison to the other technologies (incineration, land filling, land application etc.) the reed bed has a number of advantages. It is relatively inexpensive to build, operate and maintain. It consumes less energy, and discharges a minimum of CO<sub>2</sub> into the atmosphere in comparison to the other techniques of sludge disposal (Davison et al., 2005).

It has been widely accepted that reed beds have the ability to dewater and stabilise sludge (Edwards et al., 2001; Nielsen, 2003, 2005a; Nassar et al., 2006) and to reduce BOD and COD content (Gschlögl and Stuble, 2000; Davison et al., 2005; Kayser and Kunst, 2005). Additionally, removal of LAS and NPE during reed bed treatment of mesophilically digested sludge was observed during a 9 months study by Nielsen (2005b). Moreover Nassar et al. (2006) investigated the cost of sludge treatment using reed bed to be 0.34 US\$ m<sup>-3</sup> compared with 1.01 US\$ m<sup>-3</sup> for

treatment using conventional drying beds in the Gaza Strip. Therefore, reed beds are used especially in rural areas where space is relatively inexpensive for treating sludge before final disposal or use in agriculture.

Macrophytes (plants) play a critical part in the reed bed sludge treatment process, with their rhizomes creating the necessary environment for the bacterial and physical-chemical processes (Pauly et al., 2006; Nielsen, 2005a). The plant rhizomes provide surfaces for bacterial growth as well as for filtration of solids. Furthermore, their oxygen supply (Armstrong et al., 1990) creates oxidised micro-environments, stimulating both the decomposition of organic matter and the growth of nitrifying bacteria. The roots are also thought to stabilise the hydraulic conductivity at a desired level (Gumbrecht, 1992). Common reed (*Phragmites australis*), which is widely used in reed bed treatment technologies, is an aquatic grass with a distribution extending from cold temperate regions to the tropics (Karunaratne et al., 2003). It is a robust plant which can tolerate a fairly wide range of pH and salinity. However, the other aquatic grasses, bulrush (*Typha latifolia*) and reed canary grass (*Phalaris arundinacea*) are also frequently used in the reed bed systems (Vymazal, 1998, 2001). The first of the two aims of this paper is to assess the role of macrophytes in the sludge reed bed treatment technologies concerning different effects on removal of persistent organic compounds.

The second aim of this paper is to investigate dissipation kinetics as well as removal mechanism of organic micro-pollutants during the

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sludge reed bed treatment process. In contrast to an earlier study, that was conducted on a technical open sludge reed bed (Chen et al., 2009), in this study more controlled enclosed environments (container lysimeters) are being used.

Personal care product ingredients were used as marker compounds as they are among the most abundant in sludge (Kinney et al., 2006), and they are presumably emitted continuously in contrast to most other pollutants. Personal care compounds are among the most commonly detected compounds in waste water for the last 40 years (Xia et al., 2005; Kolping et al., 2002). They are released after use via sewer system into sewage treatment plants. Because of the relatively high lipophilicity of the compounds, sorption is the main process relevant for elimination in sewage treatment plants. Previous investigations have indicated that land application of sludge may be a potentially important route through which personal care products enter the environment (Xia et al., 2005). As a matter of fact, musk fragrances such as HHCB and AHTN which are mainly used in domestic purpose as well as Triclosan and OTNE have recently been identified as important anthropogenic organic contaminants in sewage sludge (Kinney et al., 2006; Simonich et al., 2002; Bester et al., 2008a,b).

HHCB, AHTN and OTNE are currently among the most frequently used fragrances in cosmetic, cleaning and personal care products,

while Triclosan is an antimicrobial agent which is widely used in toothpaste, soaps, deodorants, cosmetics and skin care lotions as well as other consumer goods (Adolfsson-Erici et al., 2002; Bester, 2005, 2007). In Table 1 the structural formulas and other details on the compounds are presented. The annual production of the respective compounds is: 350 tons Triclosan (Singer et al. 2002), over 2000 tons HHCB and AHTN (Balk and Ford, 1999; Dsikowitzky et al., 2002) and 2500–3000 tons OTNE (Gautschi et al., 2001). The primary emission route for these compounds after usage is through waste water. These are very lipophilic, persistent substances, thus they are transferred to a high extent from waste water into sludge during waste water treatment. Thus they were chosen as marker substances for elimination/degradation studies in sludge reed beds.

## 2. Materials and methods

### 2.1. Chemicals

AHTN and Triclosan were purchased from Ehrenstorfer (Augsburg, Germany) as pure compounds with purity being  $\geq 99\%$  according to the supplier. OTNE and pure standards of HHCB-lactone as well as HHCB were obtained in pure form ( $>99\%$ ) from International Flavours and Fragrances (IFF, Hilversum, Netherlands).

**Table 1**  
Compounds of interest.

Name	Formula name	CAS	Structure	M.W.	Log <sub>ow</sub>
HHCB	1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-(g)-2-benzopyran	1222-05-5		258 g mol <sup>-1</sup>	5.7
AHTN	7-acetyl-1,1,3,4,4,6 hexamethyl-1,2,3,4 tetrahydronaphthalene	1506-02-1		258 g mol <sup>-1</sup>	5.9
HHCB-lactone	HHCB-lactone	507442-53-7		272 g mol <sup>-1</sup>	4.7
Triclosan	2,4,4'-trichloro, 2'-hydroxy-diphenylether	3380-34-5		290 g mol <sup>-1</sup>	4.2–4.76
OTNE	7-acetyl, 1,2,3,4,5,6,7,8-octahydro-1,1,6,7-tetramethyl naphthalene	54464-57-2		234 g mol <sup>-1</sup>	5.7

The internal standard MX  $^{2}\text{D}_{15}$  was used to quantify the musks and OTNE as it elutes in the same fraction throughout all clean-up steps as these compounds, while TPP  $^{2}\text{D}_{15}$  was used in this experiment to quantify Triclosan and HHCB-Lactone. Musk xylene  $^{2}\text{D}_{15}$  was obtained from Ehrenstorfer (Augsburg, Germany), TPP  $^{2}\text{D}_{15}$  was synthesized from  $^{2}\text{D}_6$  phenol and phosphoroylchloride (Andresen and Bester, 2006). These internal standards were chosen as they give undisturbed signal, and also not undergo any reaction themselves.

Ethyl acetate, acetone and cyclohexane were used in analytical grade (p.a.) quality, while toluene and *n*-hexane were used in residue grade (z.R.) quality. All solvents were purchased from Merck, Darmstadt, Germany.

## 2.2. Experimental setup and sampling

In this project pre-treated sludge from a reed bed sludge treatment facility in Meppen, Germany was used. This pre-treated sludge was chosen because it is plant-compatible and capable of dewatering (Nielsen, 2003; Nassar et al., 2006).

16 containers with a size of  $1\text{ m} \times 1\text{ m} \times 1\text{ m}$  were built from stainless steel and filled with a 20 cm layer of gravel (16–32 mm) and 50 cm pre-treated sludge, from which plants and roots had been removed to prevent reeds from growing in the experiments with the other species (Fig. 1). The containers were placed outdoors on a test facility. Four containers were planted with reed canary grass (*P. arundinacea*), four with bulrush (*T. latifolia*), and another four with reed (*P. australis*) at a density of 12 plants  $\text{m}^{-2}$  to study the effects of the different species on the degradation process of organic pollutants. Four containers were left unplanted in order to distinguish the impact of the root system on the performance of the containers for the degradation of the target compounds. From May, 2007 to April, 2008 samples were taken for analysis of personal care products. In the first five months which was a very dry period, each container was watered by a water faucet for 2 min (ca. 27 l) per week to support the growing of the plants. The sludge samples were taken from a depth of 5–10 cm. The experiment was conducted under ambient temperature, water content and plant density.

## 2.3. Preparation and clean-up of sludge

After sampling, 200 g samples were immediately frozen in a refrigerating room at  $-27^\circ\text{C}$  overnight. Frozen sub-samples of 50 g wet weight were lyophilised at 2 mbar and  $-46^\circ\text{C}$  (ALPHA 1–2/LD, Christ, Osterode am Harz, Germany). The lyophilised sludge samples were

extracted by means of accelerated solvent extraction (ASE200, Dionex, Sunnyvale, USA) with ethyl acetate at  $90^\circ\text{C}$  and 150 bar. After adding an aliquot of 100  $\mu\text{l}$  internal standard solution (IS) (containing 100 ng  $^{2}\text{D}_{15}$  musk xylene and 100 ng TPP  $^{2}\text{D}_{15}$ ), the resulting extracts were concentrated by a Büchi Synchore multiport concentrator at  $80^\circ\text{C}$  and 70 mbar (Büchi, Essen, Germany) to 1 ml and were successively cleaned up with 1 g silica SPE solid-phase extraction cartridges (silica 60 obtained from Merck, Darmstadt, Germany) by elution with ethyl acetate.

These resulting solutions were condensed again and injected into a GPC-column (Biorad SX-3) ID: 2.5 cm, length: 30 cm, flow:  $5.0\text{ ml min}^{-1}$  and cyclohexane:ethyl acetate 1:1. The solvent eluting in the first 19:30 min was drained to waste, while the fraction 19:30–30:00 min was collected. Thus, macromolecules were separated as they elute in the first fraction, while sulphur, etc. are separated from the target compounds as they are eluted after the analyte fraction. The samples were finally transferred into toluene as shown above. The resulting extracts were finally fractionated on silica using 5% Methyl-*tert*-butylether MTBE in *n*-hexane and ethyl acetate successively as eluents (2 fractions).

## 2.4. Instrumental analysis

The resulting fractions were condensed and finally analysed by gas chromatography with mass spectrometric detection (GC–MS) equipped with a programmable temperature vapouriser (PTV) injector. The PTV (1  $\mu\text{l}$  injection volume) was operated in PTV splitless mode. The injection temperature of  $115^\circ\text{C}$  was held for 3 s, it was successively ramped with  $12^\circ\text{C s}^{-1}$  to  $280^\circ\text{C}$  for the transfer of the analytes. This temperature was held for 1.3 min. The injector was then ramped with  $1^\circ\text{C s}^{-1}$  to  $300^\circ\text{C}$  which was held for 7 min with 20 ml  $\text{min}^{-1}$  as a cleaning phase.

The GC separation was performed with a DB-5MS column (J&W Scientific), L: 15 m; ID: 0.25 mm; film: 0.25  $\mu\text{m}$  and a temperature programme of:  $100^\circ\text{C}$  (hold: 1 min) ramped with  $30^\circ\text{C min}^{-1}$  to  $130^\circ\text{C}$  and with  $8^\circ\text{C min}^{-1}$  successively to  $220^\circ\text{C}$ . Finally, the baking temperature was reached by ramping the column with  $30^\circ\text{C min}^{-1}$  to  $280^\circ\text{C}$  which was held for 7 min. The detector of the mass spectrometer (DSQ, Thermo Finnigan, Dreieich, Germany) was operated with 1281 V,  $230^\circ\text{C}$  ion source temperature and  $250^\circ\text{C}$  interface temperature.

## 2.5. Leachate

When rain water passes through the sludge layer, some compounds can be dissolved (mobilised from the sludge), which could also lead to a

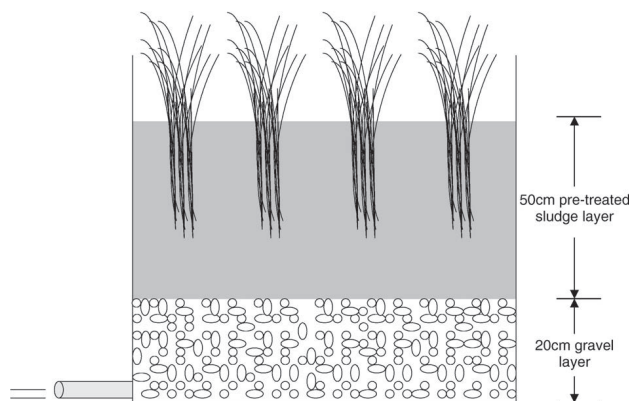


Fig. 1. Container section plan.

decreased concentration of compounds in the sludge. Thus two samples for out-flowing water were taken as duplicates from each container. 1 l samples were extracted for 20 min with 20 ml toluene by means of vigorous stirring with a teflonized magnetic stir bar after adding an aliquot of 100  $\mu$ l internal standard solution. The organic phase was separated from the aqueous one and the residual water was removed from the organic phase by freezing the samples overnight at  $-20^{\circ}\text{C}$ . The resulting extracts were then concentrated with a Büchi Synchore at  $80^{\circ}\text{C}$  and 70 mbar to 1 ml. The resulting extracts were quantified by using GC-MS.

The compounds were detected by means of their mass spectral data and retention times. For quantitative measurements the method was validated (Simonsick and Prokai, 1995; Peck, 2006; Bester, 2004, 2007, 2009). Calibrations were performed as a multi-step internal standard calibration. Recovery rates were determined by spiking sludge with respective standard concentrations at 5000  $\text{ng g}^{-1}$  (Chen et al., 2009; Chen and Bester, submitted for publication).

### 3. Results

#### 3.1. Method quality assurance

The recovery rates were 60–133%, and the relative standard deviations were 5–12%. Limits of quantification (LOQ) were calculated from the analysis of standard solutions, which gave signal to noise ratios ( $s/n$ ) of at least 10 as well as from the lowest concentration for the respective substance that was detectable from the recovery studies. Full data are given in Chen et al. (2009) and Chen and Bester (submitted for publication).

#### 3.2. Water content

Water content increased from 52% (in May 2007) to 61% (in Sep 2007) due to the 27 l of watering every week, and stayed constant after Sep 2007 since the rainfall and evaporation were equal. No significant difference of water content was detected between planted and unplanted containers as well as containers within different plants.

#### 3.3. Mineral content

The mineral content of sludge increased during the experimental period, which indicates occurrence of degradation of organic material. The mineral content ranged from 40% to 52% in the reed canary grass planted containers, from 40% to 49% in the bulrush planted containers, from 40% to 50% in the reed planted containers as well as unplanted containers. No significant difference of mineral content was detected between planted and unplanted containers as well as containers with different plants.

#### 3.4. Personal care products

The highest concentrations of the polycyclic musk compounds HHCB, AHTN were determined in the beginning of the project, which is 11,000  $\text{ng g}^{-1}$  (dry weight) HHCB and 2250  $\text{ng g}^{-1}$  (dry weight) AHTN. These are corresponding to the results obtained by Mueller et al. (2006). After 12 months, the HHCB concentrations were reduced by 25% in the reed canary grass planted containers, 27% in the bulrush planted containers, 22% in the reed planted containers and 23% in the unplanted containers. Similar to HHCB, AHTN was reduced by 24% in the reed canary grass planted containers as well as in the bulrush planted containers, 20% in the reed planted containers and 21% in the unplanted containers after 12 months.

By way of contrast, the primary metabolite of HHCB, i.e. HHCB-lactone showed increasing concentration during the experimental period. The concentration of HHCB-lactone was 1200  $\text{ng g}^{-1}$  (dry mass) at the beginning of the experiment. After 12 months the concentrations increased up to 1600  $\text{ng g}^{-1}$  (dry mass) in the reed canary grass planted

containers as well as in the bulrush planted containers, while 1700  $\text{ng g}^{-1}$  (dry mass) was reached in the reed planted and unplanted containers after 12 months.

HHCB started with concentration of 11,000  $\text{ng g}^{-1}$  (dry mass), and became 8300, 8000, 8500 and 8500  $\text{ng g}^{-1}$  (dry mass) in the reed canary grass, bulrush, reed and unplanted containers. Considering the increasing concentration of HHCB-lactone, it can be calculated that about 4%, 3%, 5% and 5% of the starting concentrations of HHCB were oxidised to HHCB-lactone in the reed canary grass, bulrush, reed and unplanted containers, respectively. However, not all of the missing HHCB turns up as HHCB-lactone. It is most probable, that the oxidation and transformation went on to form secondary metabolites. The first step is in agreement with Bester (2004), Berset et al. (2004) and Reiner and Kannan (2006) who found oxidation of HHCB to HHCB-lactone in the aeration tank of waste water sewage treatment plant.

Fig. 2a and b shows the log  $C/C_0$  plot for OTNE concentration as a function of time. The highest amount 1600  $\text{ng g}^{-1}$  (dry mass) was determined in the beginning of the project. After thirteen months the OTNE concentrations were reduced by 70%, 73%, 72% and 73% in the reed canary grass, bulrush, reed and unplanted containers, respectively.

The concentrations of Triclosan (log  $C/C_0$  plot: Fig. 3a) in the beginning of the experiment were 800  $\text{ng g}^{-1}$  (dry mass). After thirteen months the Triclosan concentrations were reduced to less than 50% and the concentrations were 360, 310, 390 and 360  $\text{ng g}^{-1}$  (dry mass) in the reed canary grass, bulrush, reed and unplanted containers. Considering a standard deviation of 12% from the method validation this difference to the starting concentration is significant.

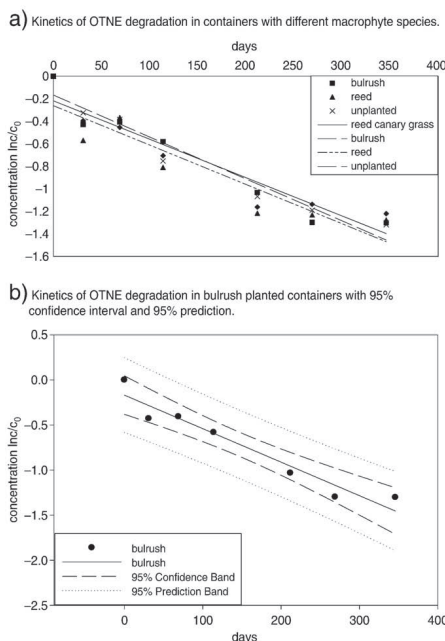


Fig. 2. Kinetics of OTNE degradation in log form. a. Kinetics of OTNE degradation in containers with different macrophyte species. b. Kinetics of OTNE degradation in bulrush planted containers with 95% confidence interval.

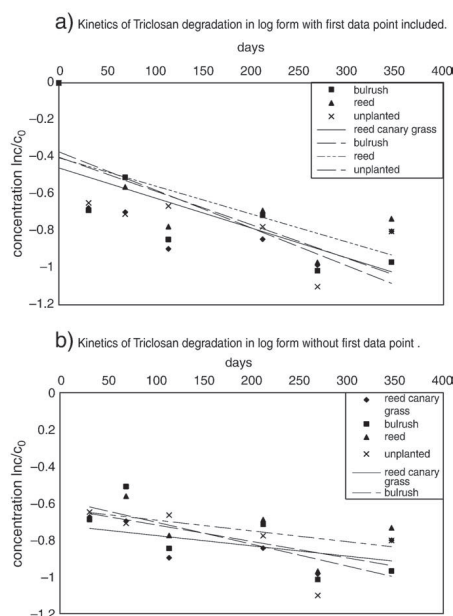


Fig. 3. Kinetics of Triclosan degradation in log form. a. Kinetics of Triclosan degradation in log form with first data point. b. Kinetics of Triclosan degradation in log form without first data point.

#### 4. Discussions

The processes that contributed to the dissipation of the studied compounds in sludge may include volatilization, plants uptake, leaching, and biological transformation (aerobic and anaerobic). Considering their generally low volatility (vapour pressure of HHCB = 0.073 Pa, AHTN = 0.061 Pa, OTNE = 0.2 Pa, and Triclosan = 0.00062 Pa), the tendency of these compounds to volatilize is low. Therefore it is expected that only small fractions of these compounds were volatilized into the atmosphere, where they could also be photolyzed (Aschmann et al., 2001; Difrancesco et al., 2004; Chen et al., 2008). To identify the relevant processes the dissipation data were analysed to determine the respective first-order reaction constants and half-lives. To discriminate microbial processes against uptake into plant biomass, the concentrations of the respective compounds were analysed in sludge as well as in plant biomass and in leachate water.

##### 4.1. Concentrations in plant biomass

To quantify the uptake of xenobiotic compounds, roots, rhizome and leaves of the three plants were analysed using the same procedure as for sludge. In these samples none of the compounds was detected (LOQ are given in Chen et al., 2009), which indicated that uptake into plant material is not relevant for the mass balance in the container experiment. This is in agreement with the results of Litz et al. (2007) who studied uptake of HHCB and AHTN by lettuce and carrots and

found HHCB and AHTN were taken up only by the carrot roots to some small extent. It is also well in agreement with the earlier field study (Chen et al., 2009).

##### 4.2. Leachate

The amount of compounds in leachate water can be calculated based on the concentration of compounds in the effluent and amount of water flowing through the system. The sludge used in this project is pre-treated sludge, which is already well dry, crumbly and structured, and not as paste like as fresh sludge and therefore it is easier for water and oxygen to pass through than for example the normal excess or dewatered sludge. In this case the worst case assumption is all rainwater and all irrigation water leach completely (no evaporation).

The rainfall amount during the experimental period (from May 2007 to April 2008) is 900 mm ( $1 \text{ mm} = 1 \text{ l m}^{-2}$ ). The irrigation volume is thus 900 l.

Since only 0.14% of the HHCB in the container was found in the leachate water, it appears that, leaching is irrelevant for the removal of compounds from the sludge in sludge reed bed treatment. The corresponding numbers are 0.16% AHTN and 0.52% Triclosan as well as OTNE (calculation details see supplementary material). Thus it occurs that (micro) biological transformation was the main dissipation mechanism for these compounds. Comparable result was obtained by Litz et al. (2007) who found a leaching rate of <0.001% for HHCB and AHTN in a leaching experiment using small soil lysimeter over a test period of 48 h simulating a rain of 200 mm.

##### 4.3. Kinetic analysis of dissipation data

Considering no significant volatilization, plants uptake and leaching occurred; biological degradation is thus dominant in the experiment process.

Biological degradation of organic compounds at low concentrations usually follows first-order kinetics, if the temperature and other critical parameters are constant. As temperature was not constant, first-order kinetics are only an approximation of the real degradation kinetics. However, this approach, with working with big installations under real conditions was assessed to give more realistic results than experiments under temperature controlled laboratory experiments. An elimination rate constant ( $k$ ) for compound removal in the reed bed containers was calculated from the concentrations during the experiment using Eq. (1). For the HHCB degradation process the respective  $k$  values are between 0.0005 and 0.0007 in the diverse experiments (canary grass, bulrush, reed and unplanted containers).

$$K = \frac{\ln \left[ \frac{C_0}{C} \right]}{t} \quad (1)$$

$k$  = elimination rate constant,  $C_0$  = starting concentration,  $t$  = time.

With Eq. (2), the half-life can be assessed:

$$t_{1/2} = \frac{\ln \left[ \frac{C_0}{C_0/2} \right]}{k} = \frac{\ln 2}{k} \quad (2)$$

Tentative half-lives were calculated for OTNE. The respective graphs are displayed in Fig. 2a. For the experiment with bulrush the results are calculated with a confidence interval as an example (Fig. 2b). OTNE was eliminated with half-lives of 204, 187, 198 and 187 days in the reed canary grass, bulrush, reed and unplanted containers, respectively (Table 2). These data are consistent with data from full scale for which half-lives of 239–277 d were found in the middle and bottom layers (Chen et al., 2009). Comparable half-lives were also observed by Difrancesco et al. (2004) with OTNE dissipation half-lives of 30–100 days in sludge-amended soils. No significant difference was detected for the respective

**Table 2**  
Tentative half-lives of the compounds during the experimental period.

Compounds	Plants	Tentative half-life (days)	95% CI
HHCB	Reed canary grass	1155	516–∞
	Bulrush	990	506–∞
	Reed	>1095	513–∞
	Unplanted	>1095	558–∞
AHTN	Reed canary grass	866	553–∞
	Bulrush	770	447–∞
	Reed	990	460–∞
	Unplanted	770	440–∞
Triclosan	Reed canary grass	433	187–∞
	Bulrush	330	175–∞
	Reed	462	201–∞
	Unplanted	385	183–∞
OTNE	Reed canary grass	204	147–330
	Bulrush	187	145–263
	Reed	198	134–378
	Unplanted	187	147–259

∞ means half-lives larger than 3 years (1095 d) also 95% confidence intervals (CI) are indicated.

setups. Obviously the support of the microbial activity by the respective plants was not significant for these experiments.

Tentative half-lives for Triclosan were calculated as continuous first-order kinetics (Fig. 3a) 433, 330, 462 and 385 days in the reed canary grass, bulrush, reed and unplanted containers, respectively (Table 2). However, the respective data could also be interpreted as a fast degradation in the first month and a slower degradation in the following ones (Fig. 3b). This behaviour could be induced by oxygen supply during installation of the experiments, or high temperatures. However degradation half-lives of 315–330 d were also found in a full scale study for the middle and bottom layers (Chen et al., 2009). No significant difference was detected for the respective setups with the different plants in the container study. Obviously the support of the microbial activity by the respective plants was not significant for these experiments. This is corresponding to the results which were gained by Ying et al. (2007) by spiking Triclosan into loamy soil with a concentration of  $1 \text{ mg kg}^{-1}$  (i.e.  $1000 \text{ ng g}^{-1}$ ), 18 days half-life was calculated under aerobic conditions within this 70 days experiment. Laboratory studies showed significant biodegradation of Triclosan in activated sludge, and indicating that adaptation was a critical factor determining the rate and extent of biodegradation (Federle et al., 2002).

Tentative half-lives for HHCB can be calculated as 1160, 990, 1390 and 1160 days in the reed canary grass, bulrush, reed and unplanted containers, respectively (Table 2). No significant difference was detected for the respective setups. Obviously the support of the microbial activity by the respective plants was not significant for these experiments. These results are consistent with data from full scale experiments in which HHCB also proved to be persistent with half-lives > three years (Chen et al., 2009).

Tentative half-lives of AHTN degradation were calculated as 870, 770, 990 and 770 days in the reed canary grass, bulrush, reed and unplanted containers, respectively (Table 2). These values should be viewed as tentatively, as the experimental period (twelve months) is too short to reveal such a slow degradation process. This also was consistent with full scale studies, in which AHTN proved to be persistent with half-lives over three years (Chen et al., 2009). No significant difference was detected for the respective setups. Obviously the support of the microbial activity by the respective plants was not significant for these experiments. Nevertheless the data indicated that HHCB and AHTN are belonging to the group of very persistent pollutant. This is corresponding to the results which were gained by Mueller et al. (2006) and Litz et al. (2007) that aerobic dissipation of HHCB and AHTN in soil/sewage sludge mixtures is very slow with half-lives of 10–17 months for HHCB and 2–24 years for AHTN. Similarly, Difrancesco et al. (2004) also found a particularly slow dissipation for HHCB and AHTN in sludge-amended soils.

#### 4.4. Effects of macrophytes

Microbial processes play a significant role for the proper functioning of reed beds. The major role of macrophytes is probably in the dewatering of sludge. The dewatering capacity of a reed bed is maintained or improved by the mechanical activity of the reeds in the sludge layer. The mechanical activity includes shoots and rhizomes, which move through the sludge, as well as the above ground movement of the plants due to wind (Nielsen, 2003, 2005a).

Also, plants provide oxygen to the sludge in the reed beds. With slow percolation of oxygen into the sludge layer, both via the reed plants and their root zone, and by diffusion through the air-sludge interface, the sludge gradually becomes oxidised and stabilised (De Maeseneer, 1997). In our experiment no significant effect of the different macrophytes on mineralisation and biodegradation of organic micro-pollutants in sludge was detected, however some other research did find positive results (Zwara and Obarska-Pempkowiak, 2000; Pempkowiak and Obarska-Pempkowiak, 2002; Nielsen, 2003, 2005a), this may be due to the low amount of sludge in the small-scale ( $1 \text{ m}^3$  box) containers, so that the influence of boundary effects and weather gain more influence on the processes than they do in full scale.

#### 5. Conclusion

The sludge reed bed container study showed that the reed bed sludge treatment technology is able to reduce persistent organic pollutant (such as HHCB, AHTN, Triclosan, and OTNE) significantly. After a twelve month experiment, only 73%–78% of HHCB, 76%–80% of AHTN, 38%–48% of Triclosan and less than 30% of OTNE were left in the containers. The decrease of pollutants during the full life time (10 years) of reed beds would be much higher than, that, obviously.

It is most likely that microbial degradation processes are the dominating ones in this setup, since most of the corresponding degradation products (metabolites) could be identified (HHCB/HHCB-lactone). An accounting of material flows in addition showed that only a small fraction (<1%) of the target substances was washed out (leached) with the effluent. The uptake of personal care products into the biomass of the macrophytes can also be neglected.

The different macrophyte species did not have a significant effect on the dewatering process as well as degradation of the respective compounds in this experiment.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.scitotenv.2009.07.023.

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### **Research paper 3:**

**Chen XJ.** and Bester K. Determination of organic micro-pollutants such as personal care products, plasticizer and flame retardants in sludge, Anal Bioanal Chem (2009) 395:1877–1884





## Determination of organic micro-pollutants such as personal care products, plasticizers and flame retardants in sludge

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**Abstract** In this study, a method for the determination of organic micro-pollutants, i.e. personal care products such as synthetic musk fragrances, household bactericides, organophosphate flame retardants and plasticizers, as well as phthalates in sludge, has been developed. This method is based on lyophilisation and accelerated solvent extraction followed by clean-up steps, i.e. solid phase extraction and size exclusion chromatography. The determination is performed by gas chromatography coupled to mass spectrometry. Stable isotope-labelled compounds such as musk xylene (MX D<sub>15</sub>), tri-*n*-butylphosphate (TnBP D<sub>27</sub>) and triphenylphosphate (TPP D<sub>15</sub>) were used as internal standards. Recovery rates were determined to be 36–114% (with typical relative standard deviation of 5% to 23%) for the target compounds. The limit of detection was 3–30 ng g<sup>-1</sup>, and the limit of quantification was 10–100 ng g<sup>-1</sup> dry matter.

**Keyword** Personal care products · Musk fragrances · Triclosan · Household bactericides · Organophosphates · Phthalates

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### Introduction

Sewage sludge is produced in waste water treatment while removing compounds causing oxygen demand (BOD<sub>5</sub>) from the waste water. Thus sludge contains high concentrations of organic matter, nutrients (nitrogen and phosphorous) and lipophilic organic micro-pollutants from the waste water. Some countries such as the Nordic countries prefer to use the nutrients in agriculture (re-cycling of sludge), while some others (e.g. Switzerland) have decided to incinerate all sludges as they prioritised to destroy all micro-pollutants. The majority of countries do a case by case decision depending on the concentrations of organic micro-pollutants and heavy metals. Thus a sound basis for analysing organic micro-pollutants in sludge is necessary to make sure that only sludge with low contaminations is used for re-cycling in agriculture. Established methods are usually single or group specific such as the methods used to analyse PAHs or PCBs [1, 2]. Often the analytical protocols are similar to those established for sediments with a high load of TOC.

The compounds included in this study were synthetic musk fragrances (musk xylene, musk ketone, HHCB, AHTN, HHCB-lactone), an antimicrobial and its metabolite (triclosan, triclosan-methyl), organophosphate flame retardants and standing for organophosphate-plasticizers (tri-*iso*-butylphosphate (TiBP), tri-*n*-butylphosphate (TnBP), tris-(2-chloroethyl) phosphate (TCEP), tris-(2-chloro-*iso*-propyl) phosphate (TCPP), tris-(dichloro-*iso*-propyl) phosphate (TDCP) and triphenylphosphate (TPP)) and the phthalate (di(2-ethylhexyl) phthalate (DEHP); Table S1). Some of these compounds have been discussed in national as well as developing EU laws on sludge as maker compounds for the re-use of this material [3, 4].

Synthetic musk fragrances are compounds used as low cost fragrances in soaps, perfumes, air fresheners, deter-

gents, fabric softeners and other household cleaning products. There are four synthetic musk fragrances accounting for 95% of the used musk. These are two polycyclic compounds (HHCB and AHTN) as well as the nitro-musks (musk xylene and musk ketone). These compounds have been detected in surface water [5, 6], in waste water [7–9] and in sewage sludge [10–12]. HHCB–lactone is the primary metabolite of HHCB (Table S1). The ratio HHCB versus its metabolite HHCB–lactone has been used to detect transformation processes of this fragrance. During the sewage treatment process, about 10% of HHCB is transformed to HHCB–lactone, which has been reported for balance assessment for polycyclic musk fragrances in a German treatment plant by Bester [8]. Reviews of several analytical strategies for the analysis of musks in sludge have been described by using accelerated solvent extraction (ASE), supercritical fluid extraction, Soxhlet extraction and liquid–liquid extraction, all of them in combination with gas chromatography–mass spectrometry (GC–MS) [13–15].

Triclosan (Table S1) is an antimicrobial agent, which is widely used in personal care products such as toothpaste, soaps, deodorants, cosmetics and skin care lotions as well as other consumer goods. Approximately 1,500 t is produced annually worldwide, and approximately 350 t of those is applied in Europe [16]. Triclosan–methyl (Table S1) is a transformation product of triclosan. These two compounds have been identified in the environment by several investigators [16–21], whereas bioaccumulation and toxicity have been studied by Orvos et al. [22], Coogan et al. [23] and De Lorenzo et al. [24]. Analytical methods for analysing antimicrobials in sludge by using GC–MS and liquid chromatography–MS have been reviewed by Peck [13].

The organophosphates included in this study were chlorinated alkylphosphates such as TCPP, TCEP and TDCP, which are mostly used as flame retardants in polyurethane. Additionally, non-derivatised alkylphosphates such as the two isomers of tri-butylphosphate (TnBP and TiBP) and TPP, which are used as plasticisers, were studied as well. Because of their relatively low cost, organophosphates especially TCPP have become the most widely used class of flame retardants [25]. These compounds are washed off from the equipped items during cleaning; the cleaning water will be discharged to the sewer and thus reach waste water treatment plants, as discussed by Fries and Puttmann [26] as well as by Meyer and Bester [27]. Additionally, these organophosphates have been detected in indoor air as well as in indoor dust by Sanchez et al. [28] and Garcia et al. [29]. Only a few analytical procedures to determine organophosphates in sludge or sediment with high TOC content have been described [30, 31].

DEHP is one of the most widely used plasticizers. It is used mainly for making PVC soft and pliable. This

plasticizer is eluted into waste water by washing and cleaning processes of the respective materials; it is assumed to have ecotoxic (endocrine disrupting) effects to aquatic organisms [32]. Because of the relatively high lipophilicity of this phthalate, sorption is the main process relevant for elimination in sewage treatment plants. Typical concentration of DEHP in sludge was found to be ranging from 10 to 100  $\mu\text{g L}^{-1}$  by Fromme et al. [33]. Extraction methods in combination with GC–MS have been described by Sablayrolles et al. [34] and Aparicio et al. [35].

The main objective of the research presented in this paper was to develop and validate an analytical multi-method to determine different classes of organic micro-pollutants such as personal care products, plasticizers and flame retardants and phthalates in sludge.

## Experimental section

### Materials

AHTN, triclosan, musk xylene, musk ketone and DEHP were purchased from Ehrenstorfer (Augsburg, Germany) as pure compounds with purities being  $\geq 99\%$  according to the supplier. Pure standards of HHCB–lactone as well as HHCB were obtained from International Flavours and Fragrances (IFF, Hilversum, Netherlands). Triclosan–methyl was synthesised from triclosan by methylation with trimethylsulfonium hydroxide solution (Macherey–Nagel, Dueren, Germany) at 40°C [20].

TCPP and TDCP were obtained from Akzo Nobel (Amersfoort, the Netherlands). These compounds were used without further purification. The technical TCPP gives three peaks in the ratio 9:3:1. In this study, only the main (first eluting) isomer was used for determination. TnBP, TiBP, TPP and TCEP were purchased from Sigma-Aldrich (Steinheim, Germany). Ethyl acetate, acetone, cyclohexane and methanol were used in analytical grade (p.a.) quality, while toluene and *n*-hexane were used in residue grade (z.R.) quality. All solvents were purchased from Merck (Darmstadt, Germany).

### Internal standards

The internal standard musk xylene D<sub>15</sub> was used to quantify the musk fragrances musk xylene, musk ketone, HHCB, AHTN, triclosan–methyl and DEHP as it elutes in the same fraction as these compounds, while TnBP D<sub>27</sub> was used to quantify TiBP, TnBP, TCEP and TCPP, and TPP D<sub>15</sub> was used in this experiment to quantify triclosan, HHCB–lactone, TDCP and TPP. Musk xylene D<sub>15</sub> and TnBP D<sub>27</sub> were obtained from Ehrenstorfer (Augsburg,

Germany); TPP D<sub>15</sub> was synthesised from D<sub>6</sub> phenol and phosphoroylchloride. These internal standards were chosen as they give undisturbed signal and also do not undergo any reaction themselves [36].

#### Analytical method

The sample preparation scheme is shown in Fig. 1: After sampling, the sludge samples were immediately frozen at -27°C overnight. “Dried sludge” such as produced at waste water treatment plants contains about 70% water; thus drying is essential to provide good wettability of the sludge with organic solvents. The frozen sub-samples of 40 g wet weight were then lyophilised overnight at 2 mbar and -46°C using an ALPHA 1-2/LD (Christ, Osterode am

Harz, Germany). The 4–6 g lyophilised sludge samples was blended with about 10 g diatomaceous earth (acid-washed obtained from MP Biomedicals, Solon, OH, USA) and homogenised in a mill (IKA A11 BASIC, Staufen, Germany) to a fine powder. The homogenates were then transferred into a 33-mL stainless steel ASE cell and extracted successively with ethyl acetate (ASE 200, Dionex, Sunnyvale, USA). After adding an aliquot of 500 µl internal standard solution (IS; containing 500 ng D<sub>15</sub> musk xylene, 500 ng TPP D<sub>15</sub> and 500 ng TnBP D<sub>27</sub>), the extract was concentrated to 1 mL by a Büchi Synchore multiport concentrator (Büchi, Essen, Germany) at 80°C and 70 mbar.

The resulting extracts were cleaned up with silica solid phase extraction (SPE) cartridges. This step is primarily protecting the next step (size exclusion chromatography (SEC)) from too many particles as well as very polar compounds. It was performed by packing 1 g of silica (silica 60 obtained from Merck, Darmstadt, Germany, predried at 105°C) into a glass column (60 mm long, 12 mm ID) with two PTFE frits on the top and bottom of silica. The silica column was conditioned with 12 mL *n*-hexane before use and eluted with 12 mL ethyl acetate after loading the samples.

The resulting extracts were again concentrated by a Büchi Synchore multiport concentrator and successively injected into an SEC system (GPC-Basix, purchased from LC-Tech, Dorfen, Germany) equipped with a glass column ID: 2.5 cm, length 30 cm, packed with 50 g SX-3 (Bio-Rad, Hercules, CA, USA). The mobile phase was cyclohexane and ethyl acetate (1:1, *V/V*) and the flow rate was 5.0 mL min<sup>-1</sup>. The solvent eluting in the first 19.30 min (97.5 mL) containing macro-molecules was drained to waste, while the fraction 19.30–30.00 min (52.5 mL) containing the analytes was collected [37]. The samples were finally transferred into toluene by adding 10 mL toluene and condensing to 1 mL. Thus, macro-molecules were separated as they are eluted in the first fraction, while sulphur, etc. are separated from the target compounds as they are eluted after the analyte fraction.

The resulting extracts were then fractionated for polarity on silica 60 using 12 mL 5% methyl-*tert*-butylether in *n*-hexane (first fraction) and 12 mL ethyl acetate (second fraction) successively as eluents. The musks, triclosan-methyl and DEHP were eluted in the first fraction, while TnBP, TnBP, TCEP, TCPP, TDGP and TPP as well as triclosan and HHCB-lactone were eluted in the second fraction according to their polarity. These fractions were transferred into toluene as described above and finally analysed by GC-MS detection.

The GC-MS system was a DSQ purchased from Thermo, Waltham, USA. The GC was equipped with a programmable temperature vapouriser (PTV) injector. The

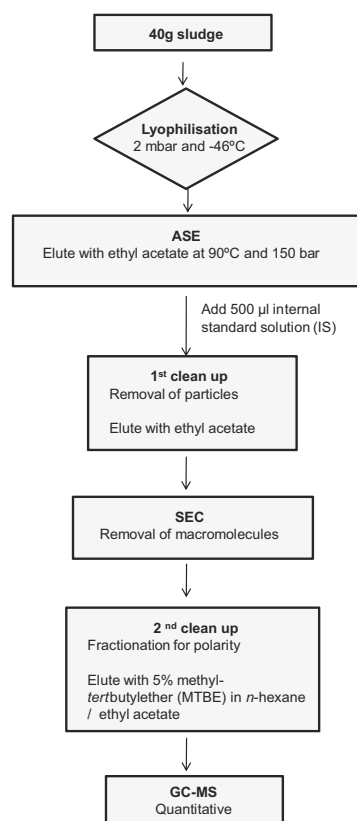


Fig. 1 Sample preparation scheme

PTV (1  $\mu$ L injection volume) was operated in PTV splitless mode. The injection temperature of 115°C was held for 3 s; it was successively ramped with 12 to 280°C s<sup>-1</sup> for the transfer of the analytes into the column. This temperature was held for 1.3 min. The injector was then ramped with 1 to 300°C s<sup>-1</sup> (open split), which was held for 7 min as a cleaning phase.

The GC separation was performed with a DB-5MS column (J&W Scientific), L was 15 m, ID was 0.25 mm, and film thickness was 0.25  $\mu$ m. The oven temperature programme started at 100°C (hold, 1 min) and was then ramped with 30 to 130°C min<sup>-1</sup> and successively with 8 to 220°C min<sup>-1</sup>. Finally, the baking temperature was reached by ramping the oven with 30 to 280°C min<sup>-1</sup>, which was held for 7 min.

The transfer line was held at 250°C, which is sufficient to transfer all compounds from the GC into the MS as the vacuum builds up in the transfer line. The ion source was operated at 230°C. Helium (4.0) was used as carrier gas with a flow rate of 1.3 mL min<sup>-1</sup>. All compounds were detected by means of their mass spectral data and retention times as shown in Table 1.

Calibrations were performed as a multi-step internal standard calibration. A stock solution was produced by dissolving 20 mg of the target compounds into 100 mL acetone. This stock solution was stored at 4°C in the dark. The weight of this flask was controlled before and after each operation. Calibration standards (3, 10, 30, 100, 300, 1,000, 3,000 and 10,000 ng mL<sup>-1</sup> in toluene) were made by serial dilution of the stock solution. The calibration standards contained the internal standards with a concentration of 100 ng mL<sup>-1</sup>. The calibration curve was calculated by using a weighted (1/*X*) linear regression.

## Results and discussions

Extracting organic compounds from sludge is optimised between extracting as much as possible of the target compound and as little as possible of the organic matter of the sludge, as the latter will be corrupting the GC or either one of the following steps.

Three experiments were performed to determine the optimal conditions for the accelerated solvent extraction in the method development and method validation after it had been decided to focus on ethyl acetate as an extractant:

1. A temperature optimisation, which was compared to total and destructive extractions
2. Validation from an artificial blank material to determine potential concentration dependency of the recovery rate as well as blank problems
3. Validation from a spiked sludge to determine recovery rates by different means as well as gain insight on realistic precision

### Optimisation of extraction temperature

Temperature is the most important parameter used in ASE extraction. ASE operates at temperatures above the normal boiling point of most solvents, using pressure to keep the solvents in the liquid phase during the extraction process. As the temperature is increased, the viscosity of the solvent is reduced, thereby increasing its ability to wet the matrix and solubilise the target analytes. However thermal degradation of the solvent or the sample might occur at higher temperatures [38, 39]. In this study a temperature range from 50 to 150°C was tested for the optimisation of

**Table 1** Retention times and selected mass fragments for the determination of the respective compounds using a DB-5 column

Compound	RT (min)	Quantifier mass (amu)	Verifier mass (amu)
OTNE	5.96	191	219
Musk xylene	8.12	282	297
Musk ketone	9.50	279	294
HHCB	8.03	243	258
AHTN	8.14	243	258
HHCB-lactone	11.73	257	272
Triclosan	11.07	288	290
Triclosan-methyl	11.30	302	304
TiBP	4.37	155	211
TnBP	5.80	155	211
TCEP	7.19	249	251
TCPP	7.46	277	279
TDCP	13.35	379	381
TPP	13.88	325	326
DEHP	14.60	149	167

extraction. For the extraction of organic micro-pollutants, one sub-sample of homogenised dried sludge was extracted by ASE with temperatures of 50, 70, 90, 110, 130 and 150°C, each followed by first clean up, SEC and the second clean up as described above. In the end the samples were measured by GC–MS. The highest concentration of HHCB, AHTN, triclosan and HHCB–lactone was found from the 70 and 90°C extractions, which is shown in Fig. 2. The increased concentration of HHCB–lactone found at 130°C was interpreted as result of an oxidation of HHCB under these conditions. Therefore, 90°C was selected as the extraction temperature because of the better extraction efficiency proved here and suggested references [38, 39]. As a control, total extractions with acetone and acidified methanol at 150°C were performed. These did not give higher concentrations than those with ethyl acetate at 90°C and 150 bar.

#### Method validation from artificial blank material (manure/soil) recovery rates and working range

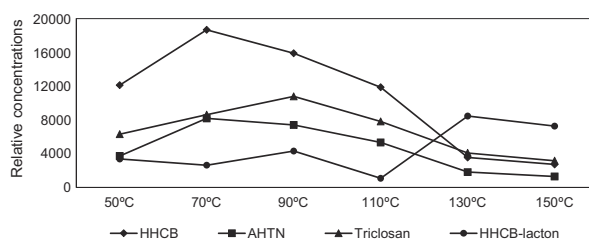
These experiments were performed to determine whether the recovery rate was dependent on the concentration or not. The working range was considered to range from the lowest to the highest concentrations for which the same recovery rates were obtained. A blank material, which contains similar TOC and ammonia content as sludge but no analytes, was produced by mixing manure from organic farming with soil (1:1). Various concentrations of the standard were spiked into the dried homogenized material. The spiked sub-samples were transferred into ASE cells, which were extracted with the method described above. Table 2 shows the recovery rate and its working range determined from the spiked artificial blank material. Figure S1 shows the recovery rate of triclosan as the function of concentration. The recovery rates for all compounds are independent on the concentrations (Table 2). It was also demonstrated that no other peaks (e.g. from decomposition/

pyrolysis) of biogenic material that could be mistaken for the analytes occurred from such matrices.

#### Method validation from spiked sludge samples (LOQ)

These recovery experiments were carried out by providing six homogeneous sub-samples from one sludge sample and each was spiked with 125 µl of the stock solution (200 µg mL<sup>-1</sup>). Two other sub-samples were left unspiked as comparison. They were lyophilised and then extracted at 90°C and 150 bar. The following sample preparation, extraction and clean up were identical to the procedures described above. For this study, dewatered digested sludge of an urban waste water treatment plant with 450,000 population equivalents, operating BOD, nitrogen and phosphorous removal was used. The sludge had a water content of 90% before lyophilisation. The mineral content of the total solid content was 33%. The concentrations of the target compounds in this sludge before and after spiking are shown in Table 3. Figure 3 shows the chromatographic characterisation of TCP in one unspiked sludge sample (18,400 ng g<sup>-1</sup>).

Since the standard deviation from this six spiked samples was low and no outlier was identified, all results were averaged. The mean recovery rates were 36–114%, and the relative standard deviations were 5–23% (Table 3), depending on the respective compounds. The lower recovery rates of musk xylene and musk ketone were possibly due to the occurrence of biotransformation of the nitro-musks during the sample preparation process [40, 41]. The limit of detection was taken as signal-to-noise ratio 3:1, and the limit of quantification (LOQ) was defined as signal-to-noise ratio 10:1, which was calculated by the Xcalibur software (Thermo, Waltham, USA) for the respective SIM chromatograms of the standard calibration (Table 3). The thus obtained LOQs are in the same range as the lower end of the working range (see above, Table 2). Comparable results were obtained by Bester [30] who used a similar procedure



**Fig. 2** Relative concentrations of HHCB, AHTN, HHCB–lactone and triclosan obtained by ASE extractions of sludge homogenates at different temperatures

**Table 2** Recovery rate and working range determined by the artificial blank material

Compound	Working range (ngg <sup>-1</sup> )	RR (%)	RSD (%)
OTNE	30–10,000	73	26
HHCB	300–10,000	87	13
Triclosan	30–10,000	88	9
TiBP	30–10,000	77	6
TCEP	10–10,000	70	11

but utilised a Soxhlet extraction to determine polycyclic musk fragrances and TCPF in waste water treatment plant.

#### Stereoisomer separation

Stereoisomer-specific determination often gives in-depth insights into ongoing processes; however, this analytical technique is more vulnerable to matrix than conventional analysis, as the respective columns have lower temperature limits. Thus, stereoisomer-specific determination requires better sample clean ups. In this study, it was tested whether the developed sample clean up is suitable also for stereoisomer determination. The gained extracts were used for stereoisomer separation of OTNE. OTNE has two chiral

centres; thus enantiomers and diastereomers may occur. The synthesis of this compound is not stereoselective; thus both kinds of stereoisomers are expected in the product [42].

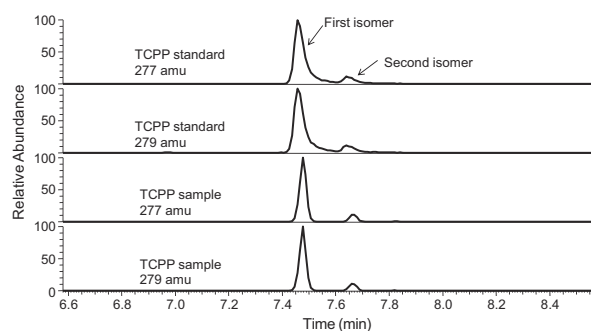
Stereoisomer separation was performed on a heptakis-(2,3-di-*O*-methyl-6-*O*-*t*-butyldimethyl-silyl)- $\beta$ -cyclodextrin (Hydrodex 6-TBDMS) column (Macherey-Nagel, Düren, Germany). This column is able to separate enantiomers as well as diastereomers of compounds such as polycyclic musks [15], but for OTNE only two major peaks were observed (Fig. 4). Thorough temperature programme and gas flow optimisation were performed and resulted in a temperature programme of 90 °C [1 min]  $\rightarrow$  10 °C min<sup>-1</sup>  $\rightarrow$  115 °C [70 min]  $\rightarrow$  10 °C min<sup>-1</sup>  $\rightarrow$  200 °C [30 min] at a constant flow of 1.2 mL min<sup>-1</sup> helium gave the best separation from the production impurities. However, only two main stereoisomers could be separated. It is thus currently unknown whether the achieved separation separates the enantiomers or diastereomers of OTNE. However, in this study, it could be demonstrated that the extracts were clean enough to give reliable stereoseparation. A multitude of standards and sludge samples were analysed in one sequence with no change of chromatographic performance. Thus this multi-method is capable to perform sample clean up for stereoseparations as well as conventional analysis.

**Table 3** Typical concentration of compounds in sludge samples, mean recovery, relative standard deviation (RSD), limit of detection (LOD) and limit of quantification (LOQ)

Compound	Concentration in unspiked sludge (ngg <sup>-1</sup> )	Calculated concentration in spiked sludge (ngg <sup>-1</sup> )	Determined concentration (ngg <sup>-1</sup> )	Mean recovery rate (%)	RSD (%)	LOD (ngg <sup>-1</sup> )	LOQ (ngg <sup>-1</sup> )
OTNE	3,000	10,927	6,513	60	6	10	30
MX	80	9,200	4,300	47	19	10	30
MK	40	7,600	2,700	36	23	3	10
HHCB	11,800	20,300	15,700	77	6	3	10
AHTN	1,600	8,900	6,100	69	5	3	10
HHCB-lactone	800	7,900	5,200	66	10	3	10
Triclosan	4,400	11,700	15,600	114	12	30	100
Triclosan-Me	70	7,300	4,000	55	10	3	10
TiBP	100	8,100	6,200	77	10	10	30
TnBP	90	7,700	4,900	64	8	10	30
TCEP	70	11,900	7,000	59	9	10	30
TCPF	18,400	28,000	27,000	96	8	30	100
TDCP	90	8,500	4,400	52	8	10	30
TPP	400	7,600	4,300	57	5	3	10
DEHP	8,700	17,200	15,000	87	21	3	10

The LOD was taken as signal-to noise ratio 3:1, and LOQ was defined as signal-to-noise ratio 10:1, which was calculated by the Xcalibur software (Thermo, Waltham, USA) for the respective SIM chromatograms of the standard calibration. Mean recovery rates were calculated by the ratio of determined concentration and calculated concentration in spiked sludge

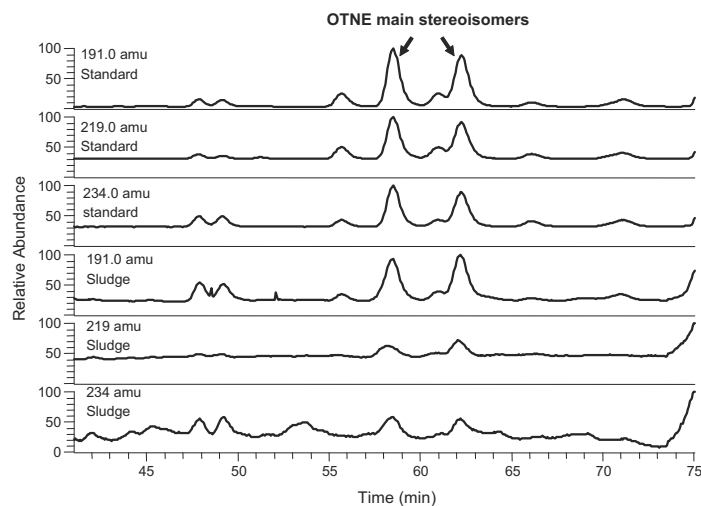
**Fig. 3** Chromatographic characterisation of the organophosphate flame retardant TCP. The third isomer was not detected as the respective SIM function was aborted before elution of this compound



### Conclusion

A precise multi-method has been developed to analyse musk fragrances, bactericides as well as organophosphates and flame retardants and phthalate by using lyophilisation, ASE in combination with the clean-up steps of SPE, SEC and the detection of GC-MS. The recovery rates obtained from two different recovery experiments performed by two different operators were comparable. In diverse projects, this method has been used to analyse several hundred sludge samples

especially in degradation and process studies, for which precision as well as stability of the system were crucial. Though the DSQ-MS needs regular cleaning of the curved prefilter quadrupole after injecting about 100 extracts in duplicate plus calibration standards, the method performed well in routine operations. It is a multi-method that in lots of cases is open to including new analytes. Also the extracts were clean enough to perform stereoseparation. Thus a method was validated, which can be the backbone of future research on organic micro-pollutants in sludge.



**Fig. 4** Separation of stereoisomers of OTNE on a heptakis-(2,3-di-*O*-methyl-6-*O*-*t*-butyldimethyl-silyl)- $\beta$ -cyclodextrin (Hydrex 6-TBDMs®) column. The main stereoisomers were detected at 58.50

and 62.27 min. Temperature programme: 90 °C [1 min]  $\rightarrow$  10 °C min<sup>-1</sup>  $\rightarrow$  115 °C [70 min]  $\rightarrow$  10 °C min<sup>-1</sup>  $\rightarrow$  200 °C [30 min] at a constant flow 1.2 mL min<sup>-1</sup>



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#### **Research paper 4:**

Bester K., **Chen XJ.**, Pauly U. and Rehfus S. Abbau von organischen Schadstoffen bei der Kläschlammbehandlung in Pflanzenbeeten, Korrespondenz Abwasser, Abfall 58 (2011) 1050-1157



# Abbau von organischen Schadstoffen bei der Klärschlammbehandlung in Pflanzenbeeten

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## Zusammenfassung

Im Beitrag werden die Versuchsdurchführung und die Ergebnisse einer Untersuchung zur Abbaubarkeit von organischen Schadstoffen, die in Klärschlämmen enthalten sind, mittels bepflanztter Beete dargestellt. Die durchgeführten Containerversuche wie auch die parallel durchgeführten Untersuchungen an den großtechnischen Klärschlammvererdungsanlagen Meppen in Niedersachsen und Kalkar-Rees in Nordrhein-Westfalen zeigen, dass bepflanzte Beete in der Lage sind, die Konzentrationen und Frachten, auch schwer abbaubare organische Schadstoffe wie den Weichmacher DEHP, das Bakterizid Triclosan sowie die Duftstoffe OTNE, HHCB, HHCB-Lacton und AHTN zu reduzieren und eine Verminderung der über den Klärschlamm in die Umwelt gelangenden Fracht von 50 % (HHCB, AHTN) bis 93 % (OTNE) zu erreichen.

Schlagwörter: Klärschlamm, Spurenstoff, anthropogen, Schadstoff, organisch, Abbaubarkeit, Elimination, Versuch, Pflanzenbeet, Fracht, Reduzierung

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## Abstract

### Degradation of Organic Pollutants in Sewage Sludge Treatment in Reed Beds

The paper describes the performance of a test and the results of a study on the degradability of organic pollutants, which are contained in sewage sludge, in reed beds. The container tests undertaken as well as the parallel tests in large-scale plants for the conversion of sewage sludge into humus, such as Meppen in Lower Saxony and Kalkar-Rees in North Rhine Westphalia, show that reed beds are able to reduce pollution levels and loads even of difficult to degrade organic pollutants such as DEHP, a surfactant, triclosan, a bactericide, as well as fragrances such as OTNE, HHCB, HHCB-lactone and AHTN and that the pollution loads that enter the environment via the sewage sludge can be reduced by between 50% (HHCB, AHTN) and 91% (OTNE).

Key words: sewage sludge, trace element, anthropogenic, pollutant, organic, degradability, removal, test, reed bed, load, reduction

## 1 Einleitung

Bepflanzte Beete werden seit 1988 in Europa für die Entwässerung von Klärschlämmen eingesetzt. Bei dem naturnahen Verfahren wird flüssiger Klärschlamm in schilfbepflanzten und zum Untergrund hin abgedichteten Beeten durch Schwerkraft (Filtration) und durch die Verdunstungsleistung der eingesetzten Pflanzen entwässert. Parallel zu den Entwässerungsprozessen findet ein Abbau eines Teils der organischen Trockenmasse durch Mikroorganismen statt. Das Endprodukt ist ein durchwurzeltes, humoses Substrat, auf das weiterhin die Bestimmungen der Klärschlammverordnung anzuwenden sind. Die Methode hat sich insbesondere für kleine und mittlere Kläranlagen im ländlichen Raum als kostengünstige und ökologische Alternative zur maschinellen Entwässerung bewährt.

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Im Rahmen des von der Arbeitsgemeinschaft industrieller Forschungsvereinigungen „Otto von Guericke e. V.“ (AiF) geförderten zweijährigen Forschungsvorhabens „Abbau von organischen Schadstoffen im Rahmen der Klärschlammvererdung“ wurden zwei großtechnische Klärschlammvererdungsanlagen in Meppen (Niedersachsen) und Kalkar-Rees (Nordrhein-Westfalen, Abbildung 1) untersucht und mit 16 eigens angefertigten, unterschiedlich beplanten Modellanlagen verglichen.

Folgende Substanzen wurden untersucht:

**OTNE** (7-Acetyl-1,2,3,4,5,6,7,8-octahydro-1,1,6,7-tetramethylnaphthalen; Handelsname Iso-E-super) hat derzeit eine weite Verbreitung in Verbraucherprodukten gefunden. 2500–3000 t dieses Duftstoffs werden jährlich verkauft [3]. Konzentrationen von 7000–30 000 ng OTNE je g Trockensubstanz (TS) Klärschlamm wurden in den USA gefunden [4], während in Europa die Konzentrationen zwischen 2000 und 4000 ng g<sup>-1</sup> lagen [5].

**Polycyclische Moschus-Duftstoffe wie HHCB** (1,3,4,6,7,8-Hexahydro-4,6,6,7,8-hexamethylcyclopenta-[g]-2-benzopyran, Handelsname zum Beispiel Galaxolid) und **AHTN** (7-Acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetrahydronaphthalen, Handelsname zum Beispiel Tonalid) werden häufig als Duftstoffe in Shampoos, Waschmitteln, Weichspülern und anderen Consumer-Produkten benutzt. [5, 6]. Beide Polycyclen haben eine geringe Wasserlöslichkeit und hohes Bioakkumulationspotenzial [7]. Die Konzentrationen dieser Substanzen in Klärschlamm aus Nordrhein-Westfalen betrugen  $3100 \pm 240 \text{ ng g}^{-1}$  (HHCB) und  $1500 \pm 150 \text{ ng g}^{-1}$  (AHTN) [8].

**HHCB-Lacton** ist der Primärmetabolit von HHCB. Während der Abwasserbehandlung werden etwa 10 % des HHCB zum HHCB-Lacton oxidiert [8]. Das Verhältnis von HHCB zu HHCB-Lacton kann im Ablauf von Kläranlagen zwischen 3 und 130 variieren. Diese Zahlen können benutzt werden, um die Oxidationseffizienz von technischen Anlagen abzuschätzen. Die Konzentrationen im Klärschlamm lagen zwischen  $600 \text{ ng g}^{-1}$  und  $3500 \text{ ng g}^{-1}$  [9].

**Triclosan** wird derzeit als Bakterizid in Zahnpasta, Mundspülwasser sowie in Funktionswäsche wie zum Beispiel Unterwäsche und Turnschuhen ebenso eingesetzt sowie zur Stabilisierung von Waschmitteln und Kosmetika [10]. Zusätzlich wird es als Polymerzusatz in Plastik-Schneidebrettern für den Lebensmittelbereich verwendet. Schätzungsweise 1500 t Triclosan werden jährlich weltweit produziert, etwa 350 t davon in Europa [11]. Triclosan zeigt eine geringe Wasserlöslichkeit und ein hohes Bioakkumulationspotenzial. In allen Klärschlammproben aus Nordrhein-Westfalen war Triclosan mit Konzentrationen um  $3000 \text{ ng g}^{-1}$  nachweisbar [12].

**DEHP** [Bis(2-ethylhexyl)phthalat] wird als Weichmacher in PVC, in Baumaterialien, aber auch in Farben und Kosmetika eingesetzt [13]. Die jährliche weltweite Produktion von DEHP liegt bei  $10^6 \text{ t}$  [14]. Die Weichmacher werden während der Lebenszeit der entsprechenden Produkte ausgewaschen und gelangen so ins Abwasser. DEHP ist eine der prioritären Substanzen der Wasserrahmenrichtlinie. Die Konzentrationen von DEHP liegen bei 1740 bis  $182\,000 \text{ ng l}^{-1}$  in Kläranlagenabläufen. In Klärschlämmen wurden 27 900 bis  $154\,000 \text{ ng g}^{-1}$  Tro-

ckenmasse und in Sedimenten 210 bis 84 400 ng g<sup>-1</sup> gefunden [15].

## 2 Material und Methoden

### 2.1. Versuchsdurchführung

Für die Versuchsdurchführung war vorgesehen, zunächst in Versuchscontainern, deren Aufbau einem Vererdungsbeet nachempfunden ist, die Abbaubarkeit der Schadstoffe zu untersuchen. Anhand der Container sollte dabei der Einfluss unterschiedlicher Wasserregimes sowie der gewählten Pflanzenart auf die Abbaubarkeit untersucht werden. Zu diesem Zweck wurden insgesamt 16 Versuchscontainer (vier Ansätze mit je vier Parallelen) auf dem Versuchsfeld der Universität Duisburg-Essen durch EKO-Plant installiert und betreut (Abbildung 2). Die Untersuchung und Auswertung der entnommenen Proben erfolgte durch das Fachgebiet Siedlungswasser- und Abfallwirtschaft der Universität Duisburg-Essen.

Die Container waren folgendermaßen ausgestattet:

Containermaße jeweils 1,0 × 1,0 × 0,95 m, unbehandeltes Stahlblech, außen mit Rostschutzlackierung versehen. An der Behältersohle wurde jeweils seitlich ein Kugelhahn aus Messing (vernickelt) zur Entnahme von Filtratproben angebracht.

In jeden Container wurden zunächst ca. 600 l maschinell entwässerter Klärschlamm mit ca. 20 % TS aus der Kläranlage Meppen eingebracht und anschließend wie folgt bepflanzt:

- Variante I: Container 1–4, bepflanzt mit Schilf (*Phragmites australis*),
- Variante II: Container 1–4, bepflanzt mit Rohrglanzgras (*Phalaris arundinacea*),
- Variante III: Container 1–4, bepflanzt mit Rohrkolben (*Typha latifolia*),
- Variante IV: Container 1–4, ohne Bewuchs.

Um den Verbleib der im Klärschlamm enthaltenen organischen Stoffe bilanziell bewerten zu können, wurden – neben Untersuchungen des Klärschlammes – Proben aus dem Filtratwasser und dem Pflanzenmaterial entnommen und auf organische Schadstoffe untersucht.

Aufgrund von Problemen mit Entwässerungsfähigkeit und Pflanzenverträglichkeit des maschinell entwässerten Klärschlammes wurden im weiteren Projektverlauf die Container entleert, mit Klärschlamm aus der Anlage Vererdungsanlage in Meppen befüllt und erneut bepflanzt. Alle Untersuchungsergebnisse beziehen sich im Folgenden auf diesen Versuchsaufbau.

### 2.2 Probenahme und Aufbereitung

Die Proben wurden mithilfe eines Stahlstechrohrs in drei Tiefenprofilen (0–20 cm, 20–40 cm, 40–60 cm) genommen. Zehn Teilproben aus den jeweiligen Schichten wurden vereinigt und in einem Stahleimer homogenisiert. Von diesen Homogenaten wurden 200 g in Glasflaschen für die Analytik versendet.

### 2.3 Durchführung der Analysen

Die instrumentelle Analyse wurde mittels GC-MS (Gas-Chromatographie mit massenspektrometrischer Detektion) durchgeführt. Hierzu wurde ein Thermo Finnigan DSQ mit einem PTV-Injektor und einem Trace Autosampler eingesetzt.

Die gaschromatographische Trennung wurde mithilfe einer DB5-MS-Säule (J&W Scientific), L: 15 m, ID: 0,25 mm, film: 0,25 µm, und eines Temperaturprogramms durchgeführt. Die verschiedenen Verbindungen wurden über ihre massenspektrometrischen Daten und Retentionszeiten identifiziert.

### 2.4 Halbwertszeiten

Da die Experimente bei wechselnden Temperaturen und Feuchtgehalten durchgeführt wurden, sind die kinetischen Daten nicht so belastbar wie zum Beispiel die aus Laborversuchen unter kontrollierten Bedingungen gewonnenen Daten. Deshalb soll im Rahmen dieses Projekts nur von einer „Abschätzung“ und nicht von einer „Bestimmung“ von Halbwertszeiten berichtet werden. Es bleibt aber hinzuzufügen, dass diese Abschätzung unter realen Bedingungen natürlich realitätsnäher ist als die unter kontrollierten Bedingungen gewonnenen Daten. Im Rahmen dieser Arbeit wird von einem Abbau erster Ordnung ausgegangen:

$$K = -\frac{\ln \left[ \frac{c_0}{c} \right]}{t}$$

Hierbei ist  $c_0$  die Startkonzentration zum Zeitpunkt  $t = 0$  und  $c$  die Konzentration zum Zeitpunkt  $t$ . Die Formel für die Halbwertszeit wird durch Umformen gewonnen:

$$t_{1/2} = \frac{\ln \left[ \frac{\frac{c_0}{2}}{c_0} \right]}{k} = \frac{\ln 2}{k}$$

## 3 Auswertung der Versuche

### 3.1 Konzentrationen der Zielsubstanzen

Die höchsten OTNE-Konzentrationen wurden zu Anfang des Experimentes mit 1600 ng g<sup>-1</sup> (TS) gefunden. Nach 13 Monaten waren die Konzentrationen bei allen Experimenten um 70 % der ursprünglichen Konzentration reduziert. Hierbei wurde kein signifikanter Unterschied der verschiedenen Bewuchsformen festgestellt (Abbildung 3).

Etwa 20 % des HHCB wurden während des Versuchs eliminiert. Die Differenz zwischen Startkonzentration und Endkonzentration beträgt etwa 3000 ng g<sup>-1</sup> (TS). Im Gegensatz dazu stieg die Konzentration des Metaboliten HHCB-Lacton um 35 % in dem Container mit Rohrglanzgras, während der Zuwachs bei dem Versuch mit Rohrkolben 32 % und bei dem mit Schilf 45 % und ohne Bewuchs 44 % (etwa 500 ng g<sup>-1</sup> (TS)) betrug. Da die Konzentrationen des Primärmetaboliten stiegen, kann davon ausgegangen werden, dass es sich bei den Eliminierungsprozessen tatsächlich um oxidative Abbauprozesse durch Mikroorganismen handelt. Es muss aber ebenfalls festgehalten werden, dass wiederum auch ein Abbau des HHCB-Lactons erfolgt, da der Verlust des HHCB insgesamt größer ist als die Zunahme des Metaboliten.

Die Konzentrationen von AHTN waren erwartungsgemäß deutlich niedriger als die des HHCB, was etwa dem derzeitigen Einsatzspektrum entspricht. Sie nahmen unabhängig von der Bewuchsform ebenfalls leicht ab, leider ist für diese Substanz derzeit kein Metabolit eines Bioabbauprozesses bekannt.

Die Triclosan-Konzentrationen nahmen innerhalb des Versuches deutlich ab (Abbildung 4). Die Startkonzentrationen ( $800 \text{ ng g}^{-1}$  (TS)) waren ausgesprochen niedrig, was nur dadurch zu erklären ist, dass das Material, das aus der seit April 2003 in Betrieb befindlichen Klärschlammvererdungsanlage Meppen stammte, bereits durch die vorherige Lagerungsdauer im schilfbepflanzten Beet in Meppen einem Vor-Abbau unterzogen war.

Ähnlich wie bei OTNE und Triclosan wurde auch für DEHP eine bedeutende Abnahme der Konzentrationen während der Versuche gefunden. Etwa 40 % Abnahme erfolgte bei der Bepflanzung mit Rohrglanzgras, während die Werte 44 % für Rohrkolben, 41 % für Schilf und 25 % für die Versuche ohne Bewuchs waren. In Bezug auf DEHP deuten sich also deutlich bessere Eliminierungen mit Pflanzenbewuchs an.

### 3.2 Massenbilanzen

Zur Sicherstellung der Messergebnisse wurde eine Massenbilanz aufgestellt (Tabelle 1), für die die Konzentrationen  $c_i$  der Zielsubstanzen im Ablaufwasser der Container bestimmt wurden.

Den Konzentrationen wurden Wassermengen aus Niederschlag (900 mm bzw. 900 l je Container) und Bewässerung (432 l je Container) gegenübergestellt und als Ablaufmenge betrachtet. Dies ist sicherlich eine Überschätzung der Ablaufmenge (Wasser), da die Betrachtung die Verdunstung des Wassers nicht berücksichtigt.

Aus diesen Ablaufmengen (Wasser) und der Konzentration in dem Ablaufwasser kann eine eluierte Menge ( $M_i$ ) der jeweiligen Substanz als die maximale Menge errechnet werden, die während des Versuchszeitraums mit dem Drainagewasser aus den Containern gespült wurde.

Aus den Konzentrationen im Schlamm  $c_s$  kann bei Berücksichtigung der Füllhöhe und der Grundfläche der Container die Menge der im Schlamm enthaltenen Substanz ( $M_s$ ) errechnet werden.

Hieraus lässt sich der relative, im Drainagewasser enthaltene Massenanteil der jeweiligen Substanz errechnen ( $M_i/M_s$ ). Er beträgt im Falle von OTNE 0,52 % des Ausgangsgehalts und ist damit vernachlässigbar. Vergleichbare Aussagen gelten für alle hier untersuchten Substanzen. Der Anteil lag zwischen 0,01 und 0,63 %. Das Auswaschen spielt infolgedessen für keine der Substanzen eine signifikante Rolle bei den Massenbilanzen oder Eliminierungen.

Zusätzlich wurde auch die grüne Blattmasse im Rahmen der Massenbilanzierung qualitativ berücksichtigt. Ein Einfluss der grünen Blattmasse auf die Reduktion der Xenobiotica (durch Aufnahme derselben in die Biomasse) kann ebenfalls ausgeschlossen werden ( $<1 \%$ ), da sowohl die gemessenen Konzentrationen in der Blattmasse sehr gering waren als auch die Blattmasse selbst gegenüber der Masse des Klärschlammes nur eine untergeordnete Rolle spielt (wenige  $\text{kg/m}^2$  gegenüber mehreren Hundert  $\text{kg Klärschlammmasse/m}^2$ ).



#### 4 Diskussion der Ergebnisse

Die durchgeführten Containerversuche wie auch die parallel durchgeführten Untersuchungen an den großtechnischen Vererdungsanlagen Meppen in Niedersachsen und Kalkar-Rees in Nordrhein-Westfalen zeigen deutlich, dass die vegetative Klärschlammbehandlung in der Lage ist, auch schwer abbaubare organische Schadstoffe zu reduzieren. Dabei handelt es sich höchstwahrscheinlich um echte Abbauprozesse, da entsprechende Abbauprodukte (Metabolite) nachgewiesen werden konnten. Eine Bilanzierung der Stoffströme zeigte ergänzend, dass nur ein Bruchteil ( $<1\%$ ) der Zielsubstanzen mit dem Filtratwasser ausgewaschen wird. Auch die Aufnahme durch die Pflanzen kann mit  $<1\%$  vernachlässigt werden. Dass die Art der Bepflanzung zudem eine untergeordnete Rolle bei den Abbauprozessen gespielt hat, kann als ein weiterer Beweis gesehen werden, dass es sich maßgeblich um substratspezifische mikrobielle Abbauprozesse handelt.

Bei den ermittelten Reduktionsraten und Halbwertszeiten ist zu berücksichtigen, dass sich diese auf Prozesse beziehen, die im schilfbepflanzten Beet allein während der rund zwölfmonatigen Trockenphase vor einer Verwertung stattfinden. Wie die niedrigen Ausgangskonzentrationen des in den Containern eingesetzten Materials aus der Anlage in Meppen zeigen, findet ein Abbau der Schadstoffe offenbar schon während der normalen Betriebsphase mit periodischer Beschlämmung – vor Einleitung einer Trockenphase – statt. Setzt man eine Betriebsdauer von fünf bis zehn Jahren bis zur eigentlichen Trockenphase vor einer Beet-Räumung voraus, in der ebenfalls schon durch das periodische Beschicken und Trockenfallen der Beete Abbauprozesse stattfinden können, wird deutlich, dass bei diesen Anlagen ein unter Umständen noch erheblich höheres Abbaupotenzial für organische Schadstoffe besteht.

Für die Gesamtbilanzierung in Hinblick auf die Umweltrelevanz ist daher eine Input-Output-Betrachtung bezüglich der Frachten hilfreich. Dazu wird die ins Beet geleitete Klärschlamm-trockenmasse (in t TM) mit den in ihr enthaltenen Zielsubstanzen (in ng/g TM) im Nassschlamm ins Verhältnis zur Masse und den Konzentrationen gesetzt, die am Ende des Behandlungszyklus zur Verwertung in die Umwelt gelangen würden. Nachfolgend ist dies am Beispiel Kalkar dargestellt (Tabelle 2), da nur hier hilfsweise die Konzentration im Nassschlamm vorlag. Die Angaben stehen unter dem Vorbehalt dieser einmaligen Stichprobe, zeigen in ihrer Tendenz aber den deutlichen Einfluss der bepflanzten Beete auf die Entfrachtung der umweltrelevanten Zielsubstanzen.

##### Beispiel Klärschlammvererdungsanlage Kalkar-Rees

Gegenüber der Input-Trockensubstanzmenge von 489 t TM befinden sich nach Abschluss der Trockenphase nur noch rund 331 t TM im Beet. Ein Großteil dieser Massenreduktion ist auf den Abbau organischer Substanz zurückzuführen. Gleichzeitig nimmt die Konzentration von der Startkonzentration  $c_0$  hin zur Endkonzentration  $c$  während des Behandlungsprozesses laufend ab. Unter Berücksichtigung einer Klärschlamm-trockenmasse von 489 t TM Input und 331 t TM Output am Ende der Trockenphase ergeben sich die in Tabelle 3 genannten Frachten.

## 5 Fazit

In den Modellanlagen kommt es während des Versuchszeitraums zu einer deutlichen Abnahme der Konzentrationen der untersuchten Xenobiotica zwischen 20 % (HHCB) und 70 % (OTNE). Aus den gewonnenen Daten wurden näherungsweise Halbwertszeiten für die Zielsubstanzen ermittelt. Die Konzentrationsabnahmen und Halbwertszeiten aus den Modellanlagen konnten für die großtechnischen Anlagen weitgehend bestätigt oder übertroffen werden (Kalkar). Da bei großtechnischen Anlagen in der Regel Trockenphasen von zwölf Monaten eingehalten werden, können die Reduktionsraten der Zielsubstanzkonzentrationen für diesen Zeitraum bei der Anlage in Kalkar wie in Tabelle 4 dargestellt angenommen werden.

Betrachtet man neben den reinen Veränderungen der Konzentrationen in der Trockenphase auch die Massenveränderung, die durch Abbau von Klärschlamm-trockenmasse eintritt, ergeben sich weitere Entlastungen der Umwelt. Am Beispiel Kalkar konnte eine Frachtverringerung der untersuchten Xenobiotica zwischen rund 50 und 93 % gegenüber der direkten Nassschlammausbringung abgeschätzt werden.

Bei den Containerversuchen zeigte sich ein untergeordneter Einfluss der eingesetzten Pflanzenarten auf die Versuchsergebnisse. Hierbei ist zu berücksichtigen, dass im Untersuchungszeitraum lediglich die Verhältnisse während Trockenphasen, ohne regelmäßige Beschlämmung, untersucht wurden. Durch ihre Verdunstungsleistung und ihre Rolle bei der Sauerstoffversorgung der Mikroorganismen sind die Pflanzen allerdings eine wichtige Voraussetzung für eine optimale Entwässerungs- und Mineralisierungsleistung großtechnischer Anlagen und somit wichtiger, unverzichtbarer Systembestandteil bei der Klärschlammbehandlung in Pflanzenbeeten. Insbesondere Schilfpflanzen tragen durch ihre Durchwurzelungsfähigkeit auch tieferer Schlammschichten dazu bei, das Gesamtsystem hydraulisch durchlässig und damit funktionsfähig zu halten.

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### Begriffsbestimmung

Die vegetative Behandlung von Klärschlämmen in bepflanzten Beeten wird häufig auch als „Klärschlammvererdung“ bezeichnet, die großtechnischen naturnahen Entwässerungsanlagen synonym als „Klärschlammvererdungsanlagen“. Diese Begrifflichkeit leitet sich aus einem von der Deutschen Bundesstiftung Umwelt (DBU) geförderten Projekt „Steigerung der Verwertung von Klärschlämmen durch verbesserte Produkte, Qualitätsnormungen und erweiterte Märkte“ [1], ab, in dem die Entwässerungsendprodukte aus schilfbepflanzten Beeten bodenkundlich charakterisiert wurden. Darin wurde festgestellt: „Der Prozess der Klärschlammbehandlung in Schilfbeeten ist bodensystematisch als ein System im Übergangsbereich zwischen Niedermoor und Mudde/Gyttja anzusprechen. Hinsichtlich der Ausgangsstoffe, Prozessbedingungen und Prozesse konnten keine systematischen Unterschiede festgestellt werden. (...) es setzen (...) Bodenbildungsprozesse ein, die das System in eine neue bodensystematische Klasse, vererdete Niedermoores, Gytjen/Mudden, überführen.“

Jordan [2] ordnet das Endprodukt der vegetativen Klärschlammbehandlung als „Anthrosol AT“ ein, also als einen unter anthropogener Beeinflussung entstandenen Boden. Da mit der Begrifflichkeit „Boden“ in der Regel die natürliche Genese des Untergrunds bezeichnet wird, wohingegen der Begriff „Erde“ zum Beispiel im landschaftsbaulichen Bereich für hergestellte Substrate steht (vgl. „Komposterde“), werden im Folgenden die Begrifflichkeiten „Vererdung“ und „Vererdungsanlagen“ beibehalten. Unabhängig von der Bezeichnung bleiben die eingesetzten Ausgangssubstanzen und Endprodukte immer Klärschlämme, die gemäß den geltenden gesetzlichen Bestimmungen zu verwerten bzw. zu entsorgen sind.

Substanz	c <sub>1</sub> Ablauf- wasser	Nieder- schlag	Bewäs- serung	M <sub>1</sub> Ablauf- wasser	c <sub>2</sub> Schlamm	Tiefe des Schlamm- beets	M <sub>2</sub> Schlamm	M <sub>1</sub> /M <sub>2</sub> relativer Massenanteil im Ablaufwasser im Vergleich zur Menge im Schlamm
	[ng L <sup>-1</sup> ]	[L m <sup>-2</sup> ]	[L]	[mg]	[ng g <sup>-1</sup> ]	[m]	[mg]	[%]
OTNE	2450	900	432	3,26	1570	0,5	630	0,520
HHCB	5430	900	432	7,23	10 100	0,5	4040	0,179
AHTN	1350	900	432	1,80	2250	0,5	900	0,200
Triclosan	1240	900	432	1,65	800	0,5	320	0,516
HHCB- Lacton	2280	900	432	3,04	1200	0,5	480	0,633
DEHP	480	900	432	6,39	11 130	0,5	4450	0,014

c<sub>1</sub> = Konzentration im Ablaufwasser, M<sub>1</sub> = Masse der im Ablaufwasser enthaltenen Substanz, c<sub>2</sub> = Konzentration im Schlamm, M<sub>2</sub> = Menge der im Schlamm enthaltenen Substanz, M<sub>1</sub>/M<sub>2</sub> = relativer Massenanteil der Substanzmenge im Ablaufwasser. Die Material-Dichte wird mit 0,8 t/m<sup>3</sup> angenommen, was großtechnischen Erfahrungen mit diesem Material entspricht.

Tabelle 1: Massenbilanzen bei den Containerversuchen

09-28.indd – Dienstag, 2. 8. 2011, 11:30 Uhr

Substanz	Nassschlamm-Input [ng/g]	letzter Messwert im Beet [ng/g]	Konzentrationsver- ringerung um
OTNE	18 000	1 600	91 %
HHCb	13 000	9 600	26 %
AHTN	2 100	1 500	29 %
Triclosan	2 400	650	73 %
DEHP	28 000	9 100	68 %

Tabelle 2: Abbau der Zielsubstanzen in der Vererdungsanlage Kalkar während eines Behandlungszyklus

Substanz	Input-Fracht [kg]	Output-Fracht (bei Verwertung) [kg]	Entfrachtung [%]
OTNE	8,8	0,6	– 93
HHCb	6,4	3,2	– 50
AHTN	1,0	0,5	– 50
Triclosan	1,2	0,2	– 83
DEHP	14	3,0	– 79

Tabelle 3: Entfrachtung der Klärschlammerte Kalkar

Substanz	Abgeschätzte Halbwertszeit [Tage]	Konzentrationsbezogene Eliminationsrate in zwölf Monaten
OTNE	156	77 %
HHCb	924	24 %
AHTN	492	35 %
Triclosan	205	65 %
DEHP	250	55 %

Tabelle 4: Abgeschätzte Halbwertszeiten und rechnerisch ermittelte konzentrationsbezogene Eliminationsraten der untersuchten Xenobiotica in 12-monatiger Trockenphase (Kalkar)



Abb. 1: Klärschlammvererdungsanlage Hönnepel (acht Beete, 540 t TS) (Foto: EKO-Plant)



Abb. 2: Die 16 Versuchscontainer auf dem Testgelände (Foto: EKO-Plant)

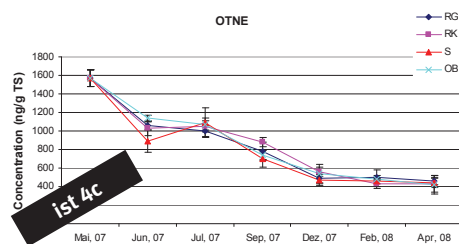


Abb. 3: Konzentrationen von OTNE im Containerversuch zur Schlammvererdung [ng g<sup>-1</sup> TS]; RG: Rohrglanzgras, RK: Rohrkolben, S: Schilf, OB: ohne Bewuchs

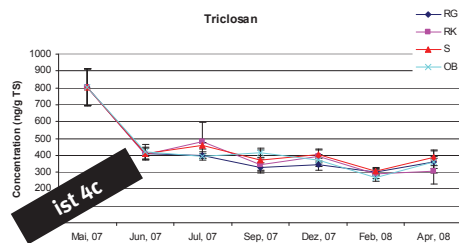


Abb. 4: Konzentrationen von Triclosan im Containerversuch zur Schlammvererdung [ng g<sup>-1</sup> TS]; RG: Rohrglanzgras, RK: Rohrkolben, S: Schilf, OB: ohne Bewuchs



### **Research paper 5:**

**Chen XJ.**, Nielsen JL., Furgal K., Liu YL., Lolas IB. and Bester K. Elimination of triclosan and formation of methyl-triclosan in activated sludge under aerobic conditions, Chemosphere 84 (2011) 452–456







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## Biodegradation of triclosan and formation of methyl-triclosan in activated sludge under aerobic conditions

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### ABSTRACT

Triclosan is an antimicrobial agent which is widely used in household and personal care products. Widespread use of this compound has led to the elevated concentrations of triclosan in wastewater, wastewater treatment plants (WWTPs) and receiving waters. Removal of triclosan and formation of triclosan-methyl was investigated in activated sludge from a standard activated sludge WWTP equipped with enhanced biological phosphorus removal. The removal was found to occur mainly under aerobic conditions while under anoxic (nitrate reducing) and anaerobic conditions rather low removal rates were determined. In a laboratory-scale activated sludge reactor 75% of the triclosan was removed under aerobic conditions within 150 h, while no removal was observed under anaerobic or anoxic conditions. One percent of the triclosan was converted to triclosan-methyl under aerobic conditions, less under anoxic (nitrate reducing) and none under anaerobic conditions.

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### 1. Introduction

Triclosan (2,4,4'-trichloro-2'-hydroxydiphenylether) is currently used as a bactericide in personal care products such as toothpaste, shampoos, and soaps. It is additionally used as a stabilizing agent in a multitude of detergents and cosmetics (Adolfson-Erici et al., 2002). Triclosan inhibits bacterial growth by blocking lipid biosynthesis (Schweizer, 2001). Microalgae communities are particularly sensitive to triclosan with effective concentrations around 10 ng L<sup>-1</sup> (Wilson et al., 2009). A mechanism responsible for this effect has been proposed (Franz et al., 2008). Additionally, triclosan has also been linked to a range of health and environmental effects, such as skin irritation, allergy susceptibility, and also other ecological toxicity to the aquatic and terrestrial environment (Coogan et al., 2007), e.g. it has an effect on earth worms (*Eisenia fetida*) (Lin et al., 2010) and on Japanese medaka fish (Nassef et al., 2010).

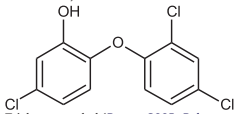
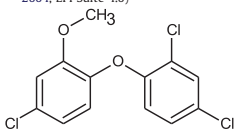
After use triclosan ends up in the wastewater with typical concentrations of 1–10 µg L<sup>-1</sup> (Adolfson-Erici et al., 2002; Lindström et al., 2002; Singer et al., 2002; Bester, 2005). Removal of about 90% was measured in wastewater treatment plants (WWTP) employing conventional activated sludge process of which 40–60% was due to biodegradation while the remainder was due to sorption to the sludge (Singer et al., 2002; Bester, 2003, 2005; Coogan et al., 2007; Heidler and Halden, 2007; Ying et al., 2007).

On the other hand, this means that most removal occurs due to biodegradation processes (Singer et al., 2002; Bester, 2003; Heidler and Halden, 2007). However, only little is known about the reaction pathways and conditions (Federle et al., 2002). About 5% of triclosan is biomethylated to triclosan-methyl (2,4,4'-trichloro-2'-methoxy-diphenylether) (Bester, 2003, 2005; Heidler and Halden, 2007). The structural formulas and basic physico-chemical parameters of triclosan and triclosan-methyl are compared in Table 1. Another 5% of triclosan is transformed to bound residues (Bester, 2003). The biochemical pathways and conditions for formation of triclosan-methyl are largely unknown up to now, as most studies focused on the mass flow of triclosan-methyl in the WWTP treatment process (Bester, 2005), its formation in estuarine systems (DeLorenzo et al., 2007) as well as bioaccumulation of triclosan-methyl in fish samples (Lindström et al., 2002; Balmer et al., 2004). It is known, though, that triclosan-methyl is more persistent, lipophilic, bio-accumulative and less sensitive towards photo-degradation in the environment than its parent compound (Lindström et al., 2002; Balmer et al., 2004). Typical concentrations of triclosan in sludge were 2–8 mg kg<sup>-1</sup> dry matter in Germany (Bester, 2003) while triclosan-methyl was only detected with 0.004–0.311 mg kg<sup>-1</sup> (dry weight) in sewage sludge samples from municipal wastewater treatment plants in Spain (Sánchez-Brunete et al., 2010).

To maximize the biodegradation of compounds like triclosan and triclosan-methyl it is crucial to understand by which process and in which part of the treatment plants triclosan is eliminated and by which process triclosan-methyl is generated. There are

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**Table 1**  
Structural formulas and other environmental parameters of triclosan and triclosan-methyl.

<p>Triclosan (Bester, 2005; Lindström et al., 2002, EPI Suite 4.0)</p>  <p>Triclosan-methyl (Bester, 2005; Balmer et al., 2004, EPI Suite 4.0)</p> 	<p>Name: 2,4,4'-trichloro, 2'-hydroxy-diphenylether Water solubility: 4.621 mg L<sup>-1</sup> Vapor pressure: 0.00062 Pa log Kow: 4.2–4.8 log Koc: 4.3 pKa: 7.9</p> <p>Name: 2,4,4'-Trichloro, 2'-methoxy-diphenylether Water solubility: 0.4 mg L<sup>-1</sup> Vapor pressure: 0.00093 Pa log Kow: 5 log Koc: 4.1</p>
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three basic processes in biological treatment in the WWTP: aerobic, anaerobic and anoxic. Aerobic (oxygen present) biological treatment is generally used removal of BOD (biochemical oxygen demand) as well as for nitrification (ammonia to nitrate). Anoxic conditions (no oxygen but nitrate present) are used for denitrification (nitrate to nitrogen gas). True anaerobic conditions (neither oxygen nor nitrate present) are limited to sludge digestion processes such as methane production. These three individual types of biological treatment processes can be run in one tank with different operating regimes in time or in separate tanks to offer better treatment. The current study compared the degradation of triclosan and formation of methyl-triclosan under the different conditions in laboratory-scale experiments to determine which of the processes were important for the biodegradation of triclosan in waste water treatment.

## 2. Materials and methods

### 2.1. Activated sludge sampling

Activated sludge samples for the preliminary experiments were sampled from Aalborg East wastewater treatment plant (WWTP), which processes  $6 \times 10^6$  m<sup>3</sup> wastewater (100 000 population equivalents, PE) annually. Samples for the detailed aerobic experiments were from Aalborg West WWTP, which processes  $22 \times 10^6$  m<sup>3</sup> wastewater (330 000 PE) annually.

The other key parameters of the plants are similar. Both receive about 80% municipal wastewater and 20% from local industries. They run with a hydraulic retention time of 24–30 h and sludge retention time of 25–30 d, and the process configurations include a screen chamber, primary sedimentation basins, activated sludge treatment basins and a final clarifier before the treated water is released into the Limfjord. Nitrification and denitrification are performed as alternating denitrification. Phosphorous removal is performed mostly by biological means. The suspended solids (SS) content of the activated sludge was 4 g L<sup>-1</sup> and its volatile solids content was 2.5 g L<sup>-1</sup> during the sampling period.

### 2.2. Degradation experiments

Biodegradation experiments were carried out in 5 L glass bioreactors. During the experiments, all reactors were maintained at  $17 \pm 2$  °C. The reactors were completely covered by aluminium foil to prevent photolytic degradation. They were monitored daily for loss of water by weighing, eventual loss of water was compensated

by adding tap water. No action was undertaken to prevent volatilization of triclosan, as the vapor pressure of triclosan and triclosan-methyl, both are very low (Table 1). The reactors were stirred by means of teflonized magnetic stir bars to keep the sludge homogeneous. No additional carbon source was added to the system, thus they were run as static reactors. Duplicated sludge samples were taken every day from each reactor.

The incubation conditions were established as:

- (1) *Aerobic conditions* by supplying air through a diffuser stone with a flow rate of 1.3 L h<sup>-1</sup>.
- (2) *Anaerobic conditions* were maintained by constantly flushing the respective bioreactor with nitrogen gas.
- (3) *Anoxic* (nitrate reducing) conditions were maintained by constant addition of potassium nitrate (KNO<sub>3</sub>)(44 gN d<sup>-1</sup> L<sup>-1</sup>).

The preliminary experiments were incubated for 80 h under aerobic, anaerobic and anoxic conditions with starting concentrations of 0.1 mg L<sup>-1</sup> triclosan, which is exceeding typical wastewater concentrations by a factor of 10 but it is in the same range as expected in activated sludge in municipal WWTPs (Bester, 2005).

Detailed aerobic experiments were performed for 10 d at five different initial triclosan concentrations to determine the rate of triclosan-methyl formed from triclosan under aerobic conditions. Triclosan concentrations of 0.02, 0.5, 1, 2 and 3 mg L<sup>-1</sup> were used in order to investigate whether the degradation of triclosan and formation of triclosan-methyl were concentration related. The 3 mg L<sup>-1</sup> is towards the very high end of the concentration that can still be found in rare cases in sludge (Stasinakis et al., 2007). The high concentrations were chosen, to be able to discriminate between triclosan and triclosan-methyl already present in the sludge and those freshly spiked for the experiments. In these experiments oxygen concentrations were measured and continuously kept above 4.0 mg L<sup>-1</sup>.

### 2.3. Extraction and instrumental analysis

#### 2.3.1. Liquid sludge

Ten millilitre sludge samples from the experiments were diluted by tap water to 1 L and extracted successively for 20 min with 10 mL toluene by means of vigorous stirring with a teflonized magnetic stir bar after adding an aliquot of 100 µL of internal standard solution (musk xylene D<sub>15</sub>). The organic phase was separated from the aqueous one and the residual water was removed from

**Table 2**

Quality assurance data including the MS data (analytical and verifier ions) as well as limit of quantification (LOQ) of the experiments for the liquid samples. Data for recovery rate (RR) and relative standard deviation (RSD) for liquid sludge were from Bester (2003), while those for solid sludge were from Chen and Bester (2009).

Analyte	Analytical ion (amu)	Verifier ion (amu)	Retention time (min)	LOQ (ng L <sup>-1</sup> )	(RR) for liquid sludge (%)	(RSD) for liquid sludge (%)	(RR) for solid sludge (%)	(RSD) for solid sludge (%)
Triclosan	288	290	6.11	10	88	11	114	12
Triclosan-methyl	302	304	6.04	0.3	102	11	55	10

the organic phase by freezing the samples overnight at -20 °C. The organic extracts were concentrated to 1 mL with a nitrogen flow condenser at 55 °C.

### 2.3.2. Solid sludge

To determine sorption of triclosan into the solid phase, another 10 mL sludge samples were taken every day from each reactor. The samples were filtered through GC-50 glass fiber filters (Advantec, Tokyo, Japan) with pore size of 0.2 µm. Filter residues (sludge solid matter) were immediately stored in a refrigerating room at -27 °C overnight and then lyophilized at 2 mbar and -46 °C. The lyophilized samples were extracted by means of accelerated solvent extraction (ASE) with ethyl acetate at 90 °C and 150 bar (Chen and Bester, 2009). The resulting extracts were then concentrated by using a Büchi multiport concentrator at 80 °C and 70 mbar (Büchi, Essen, Germany) after adding 10 mL toluene and 100 µL internal standard solution.

### 2.3.3. Instrumental analysis

Triclosan extracts from the liquid and solid sludge samples were both finally analysed by gas chromatography with mass spectrometric detection (GC-MS, Thermo-Trace GC-MS) equipped with a splitless injector and A200S autosampler. Samples (1 µL) were injected into the injector in splitless (1.5 min) mode held at a temperature of 240 °C. The GC separation was performed with a Rxi-5Sil MS column (Restek, Bellefonte, USA), L: 10 m; ID: 0.18 mm; film: 0.18 µm and a temperature programme of: 90 °C (hold: 1 min) ramped with 50 °C min<sup>-1</sup> to 135 °C and then with 10 °C min<sup>-1</sup> to 220 °C. Finally, the baking temperature was reached by ramping the column with 40 °C min<sup>-1</sup> to 260 °C which was held for 6 min. Helium (5.0) was used as carrier gas with a flow rate of 1.3 mL min<sup>-1</sup>. The transfer line of the mass spectrometer (Trace MS, Thermo Finnigan, Dreieich, Germany) was held at 250 °C. The ion source was operated at 160 °C. The mass spectrometer was operated in selected ion monitoring (SIM) utilizing 31–61 ms dwell time. The detector of the mass spectrometer was operated at 450 V. Table 2 lists the retention times of triclosan and triclosan-methyl and the mass fragments used for the detection.

### 2.3.4. Data treatment

The average of the duplicate extractions measured by duplicate injections was used for further data processing. The calibrations were performed as a multi-step internal standard calibration (10–10 000 ng mL<sup>-1</sup>). The full method and validation data for triclosan and triclosan-methyl for liquid samples were described in Bester (2005), while those for the solids were described by Chen and Bester (2009). Both are shown in Table 2. To additionally validate this method for recovery of triclosan from liquid sludge, it was tested by extracting several activated sludge samples spiked with this biocide. Five different concentrations (between 20 µg g<sup>-1</sup> and 3000 µg g<sup>-1</sup>) were dosed and for each concentration two samples were extracted; thus 10 extractions were performed. The recovery rate of triclosan was 82% with 10% relative standard deviation, which is consistent with previous measurements (Bester, 2005).

## 2.4. Materials

Triclosan was purchased from Ehrenstorfer (Augsburg, Germany) with a purity of ≥99% according to the supplier. Triclosan-methyl was synthesized from triclosan by methylation with trimethylsulfonium hydroxide solution (Macherey-Nagel, Dueren, Germany) at 40 °C (Bester, 2003). Toluene was used in residue grade (z.R.) quality and purchased from Merck (Darmstadt, Germany). The internal standard musk xylene D<sub>15</sub> (Ehrenstorfer, Augsburg, Germany) was used to quantify triclosan and triclosan-methyl (Andresen and Bester, 2006).

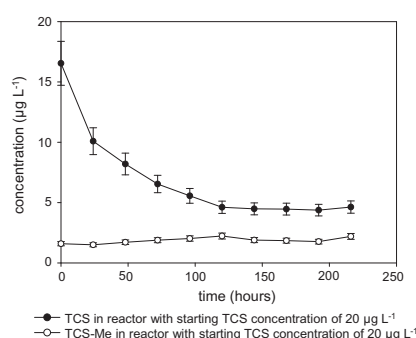
## 3. Results and discussions

### 3.1. Preliminary experiments

In this experiment the fate of triclosan was investigated in reactor experiments under aerobic, anaerobic and anoxic conditions with sludge from Aalborg East WWTP. After 80 h the concentration of the parent compound was reduced from 30 to 15 µg L<sup>-1</sup> (49%, i.e. significantly) under aerobic conditions, but only from 32 to 28 µg L<sup>-1</sup> (11%) and from 32 to 29 µg L<sup>-1</sup> (16%) under anaerobic and anoxic conditions, respectively, which is very close to the method standard deviation, i.e., 11% (Bester, 2005).

Opposite to the triclosan concentrations, those of triclosan-methyl concordantly increased from 4.2 to 5.0 µg L<sup>-1</sup> (16%) during the aerobic incubation and from 4.1 to 4.8 µg L<sup>-1</sup> (17%) during the anoxic incubation. Considering the analytical standard deviation, this increase is significant. Additionally, no change of concentrations was detected under anaerobic condition.

In summary, the fastest removal triclosan removal and its highest transformation rate to triclosan-methyl were determined under aerobic conditions. Therefore, the more detailed experiments on



**Fig. 1.** Concentrations of triclosan and triclosan-methyl in aerated reactors. Starting concentration 20 µg L<sup>-1</sup> triclosan (unspiked). Error bars indicate standard error of 11% (Bester, 2005).

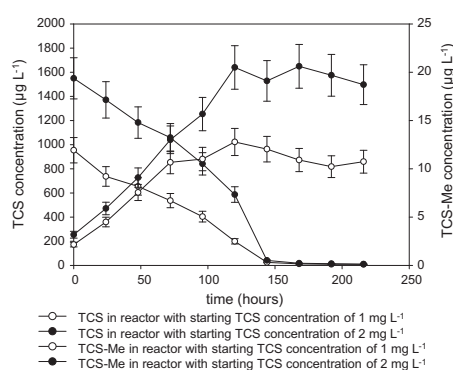


Fig. 2. Concentrations of triclosan and triclosan-methyl in aerated reactors. Starting concentration of 1 and 2 mg L<sup>-1</sup> triclosan (spiked). Error bars indicate standard error of 11% (Bester, 2005).

degradation and methylation of triclosan were carried out in activated sludge under the aerobic conditions.

### 3.2. Detailed aerobic kinetic experiments

To make sure the elevated concentrations of triclosan-methyl at the end of the experiment really originated from the freshly added triclosan and not from an old and eventually unknown pool of triclosan in the sludge, several experiments were performed with different triclosan concentrations in aerobic experiments. Triclosan concentrations were rapidly reduced in all reactors while triclosan-methyl concentrations increased concomitantly. Fig. 1 shows these data for the reactors with 0.02 mg L<sup>-1</sup> starting triclosan concentrations (unspiked), while in Fig. 2 the data for starting triclosan concentrations of 1 and 2 mg L<sup>-1</sup> (spiked) are shown, respectively. The major part of removal was achieved within 150 h (Figs. 1 and 2), after which the triclosan concentrations remained almost constant at less than 0.01 mg L<sup>-1</sup> to the end of the experiment (220 h) (Fig. 1).

The production of triclosan-methyl occurred in all experiments. The concentrations of triclosan-methyl increased according to the starting levels of the parent compound, though the concentrations of the metabolite remained significantly lower than the initial parent concentrations (Fig. 2). It is assessed that in these reactors 1% of triclosan was transformed into triclosan-methyl during the experiment under aerobic conditions. However, in the reactor with triclosan starting concentration of 0.02 mg L<sup>-1</sup> (Fig. 1), the production of

triclosan-methyl was mostly obscured by the background concentrations (from the sludge from the waste water treatment plant). In the reactors fed with 1 and 2 mg L<sup>-1</sup> triclosan, the concentrations of triclosan-methyl increased (Fig. 2), reaching the highest concentrations after 120 h, at which they remained until the end of the experiment. The concentration increase of the metabolite coincided with the concentration decrease of the parent compound. Though no strict mathematical equations could be established, it is clear, that the higher the starting concentration of triclosan was, the higher was the metabolite concentration at the end of the experiment, thus proving the triclosan-methyl was really formed from the added triclosan. The experiment thus indicates that the biomethylation of triclosan can occur in aerobic reactors. As the concentrations of triclosan-methyl are unchanged even after more than 100 h after the main pool of triclosan is consumed, it is obvious that the metabolite cannot be degraded within timeframes relevant for wastewater treatment.

To quantify the possible sorption of triclosan, solid samples were analysed. Consistently 10% of the triclosan found in the experiment medium (liquid sludge) was sorbed to the solids throughout the experiment. The triclosan concentrations in the solid phase show thus decrease in parallel to those in the liquid phase. The partitioning of triclosan between the solid and liquid phase remains constant, thus exchange processes are quick in comparison to the degradation. Additionally, the pH value of the sludge was measured as triclosan adsorption and extraction are pH dependent (Lindström et al., 2002). The pH value remained constant (6.9 ± 0.5) during the experiment indicating that the concentration changes measured are not influenced by pH.

At low concentrations (normal WWTP levels, up to 20 µg L<sup>-1</sup>) the biological degradation of triclosan followed the first-order kinetics (Fig. 1), while the reaction kinetics is more complex at higher concentrations (>500 µg L<sup>-1</sup>). Thus, the pseudo-first-order rates and half-lives from reactors were calculated to give an overview of the performance of the system (Table 3). The estimated half-lives ( $t_{1/2}$ ) were found to be 54–86 h, and the elimination rates considering a 10-d period were 75% and 86% for the reactors with initial triclosan concentration of 0.02 and 0.5 mg L<sup>-1</sup>, and 99% for reactors with the initial triclosan concentration of 1, 2 and 3 mg L<sup>-1</sup>.

The half-life of triclosan in this experiment was not dependent on the concentration. However, the elimination rates were relatively lower when the starting concentration was low (0.02 mg L<sup>-1</sup>), and reached higher values (>99%) when the starting concentration was high (>1 mg L<sup>-1</sup>). These data are from steady state lab scale experiment, thus should be extrapolated to full-scale WWTPs (which are flow through systems) with caution, as external carbon sources, temperature, interference of other organic compounds etc. may lead to different rates.

The rate constants of triclosan-methyl generation increased concordantly with the starting concentration of triclosan as shown in Table 3. With the initial triclosan concentrations of 0.5, 1, 2 and

Table 3

Degradation rate constant ( $k$ ) of triclosan and generation rate of triclosan-methyl in different concentrations in aerobic activated sludge systems. Starting concentration ( $C_0$ ) and final concentrations ( $C_f$ ) are given. The final concentrations were measured after 220 h. Half-lives and pseudo-first-order rate constants for the degradation of triclosan were calculated from the data between lag phase (24 h) and end of reaction (120 h), whereas generation rates of triclosan-methyl were calculated by using the data between 0–120 h.

Removal of TCS					Formation of TCS-Me		
$C_0$ (µg L <sup>-1</sup> )	$C_f$ (µg L <sup>-1</sup> )	$k$ (s <sup>-1</sup> )	$R^2$	$t_{1/2}$ (h)	$C_0$ (µg L <sup>-1</sup> )	$C_f$ (µg L <sup>-1</sup> )	$k$ (s <sup>-1</sup> )
20	5	0.0081	0.9969	86	1.6	2.2	0.004
500	55	0.0095	0.9961	73	2.8	6.4	0.0054
1000	10	0.0129	0.8832	54	2.2	10.7	0.0103
2000	10	0.0085	0.9451	82	3.2	18.7	0.0127
3000	10	0.0123	0.7094	56	3.2	19.8	0.0129

\* This experiment was ended after 168 h.

3 mg L<sup>-1</sup>, the rate constants were 0.0054, 0.0103, 0.0127 and 0.0129 s<sup>-1</sup>, respectively.

Biomethylation of triclosan under aerobic conditions was surprising as methylation of pollutants such as mercury (Gray et al., 2004, 2006; Barringer and Szabo, 2006), antimony, arsenic (Duster et al., 2008), bismuth (Michalke et al., 2002) and phenols (Pfeifer et al., 2001) is usually associated with anaerobic, anoxic (no oxygen but nitrate present), methanogenic or sulfate reducing regimes. However, biomethylation, e.g., by cobalamin (Vitamin B12) (Wehmeier et al., 2004) is not restricted to anaerobic conditions. Older literature reported the conditions that induced methylation processes were rather “organic-rich” (Compeau and Bartha, 1985), while others have reported that polychlorinated phenoxy phenols (PCPP) were biomethylated in contaminated soil and in several pure and mixed bacterial cultures under aerobic conditions (Valo and Salkinoja-Salonen, 1986). Additionally, biomethylation of chlorinated phenolic compounds (Häggblom et al., 1988) and tetrabromobisphenol-A (George and Häggblom, 2008) has been detected under aerobic conditions.

#### 4. Conclusions

Triclosan-methyl was formed concomitantly with the removal of triclosan in activated sludge under aerobic conditions. Triclosan-methyl was also formed under anoxic (nitrate reducing) conditions although at lower rates but was not formed under anaerobic conditions in laboratory experiments. According to these laboratory experiments, the emissions of triclosan-methyl will thus be affected mostly by the management of the BOD removal and nitrification tanks but not during anaerobic digestion.

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## Ozonation products of triclosan in advanced wastewater treatment

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### ABSTRACT

Triclosan is an antimicrobial agent widely used in many household and personal care products. Widespread use of this compound has led to the elevated concentrations of triclosan in wastewater, wastewater treatment plants and receiving waters. In this study removal of triclosan by aqueous ozone was investigated and the degradation products formed during ozonation of an aqueous solution of triclosan were analyzed by GC-MS and HPLC-MS/MS. The following transformation products have been identified: 2,4-dichlorophenol, chlorocatechol, mono-hydroxy-triclosan and di-hydroxy-triclosan during treatment process. Cytotoxicity and genotoxicity of pure triclosan and 2,4-dichlorophenol have been investigated and the results showed reduced genotoxic effects after ozonation, though the respective chlorophenol is harmful to aquatic organisms.

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## 1. Introduction

Triclosan (2,4,4'-trichloro-2'-hydroxydiphenylether, CAS: 3380-34-5) is currently used as an antimicrobial agent in toothpaste, mouthwash, liquid soap and in functional clothing such as functional shoes and underwear (Engelhaupt, 2007). It is also used as a stabilizing agent in a multitude of detergents and cosmetics and as an antimicrobial agent in polymeric food cutting boards (Adolfsson-Erici et al., 2002; Dann and Hontela, 2011). Approximately 1500 t are produced annually worldwide, and approximately 350 t of those are applied in Europe (Singer et al., 2002). The primary emission route for triclosan after usage is through wastewater. In fact, investigators have

detected triclosan in numerous municipal wastewater influent samples at concentrations in the range of 0.5–4.5 µg L<sup>-1</sup> (Buth et al., 2011; Lindström et al., 2002). In wastewater treatment plants (WWTPs) 90% of the incoming triclosan was removed from the water (Bester, 2003, 2005; Heidler and Halden, 2008; Singer et al., 2002), which is a high but not complete removal. As a result, it has been found in some sewage treatment plant effluents as well as in surface water and ground water in many countries (Adolfsson-Erici et al., 2002; Balmer et al., 2004; Bester, 2005). In addition, it has been detected in fish, soil and sediments due to its hydrophobicity (Coogan et al., 2007; Lozano et al., 2010; Xie et al., 2008).

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Triclosan inhibits bacteria growth by blocking biosynthesis of lipids, which is necessary for building cell membranes and reproduction (Levy et al., 1999; Schweizer, 2001). The impact of triclosan on aquatic organisms and the ecosystem in general has been investigated in several *in vivo* studies. These studies showed that triclosan is toxic to fish (Lindström et al., 2002), algae (Wilson et al., 2003) and other aquatic organisms (Orvos et al., 2002). Algal toxicity was shown at a minimum concentration of  $0.15 \mu\text{g L}^{-1}$  for up to 13 days of exposure and community changes were visible even at  $0.015 \mu\text{g L}^{-1}$  (Wilson et al., 2003). The  $\text{EC}_{50}$  of triclosan in fish is between 240 and  $410 \mu\text{g L}^{-1}$  (Lindström et al., 2002; Orvos et al., 2002). *In vitro* studies on human gingival cells reported toxic effects at concentrations between  $4.3$  and  $28.96 \text{ mg L}^{-1}$  depending on the test and the exposure time (Zuckerbraun et al., 1998). Acute toxic effects were found to start at  $0.28 \mu\text{g L}^{-1}$  using the bioluminescent bacteria *Vibrio fischeri* (Farre et al., 2008). In addition to these toxic effects it was reported that triclosan and its degradation products show endocrine disrupting effects (Foran et al., 2000; Ishibashi et al., 2004; Raut and Angus, 2010). The toxicity of 2,4-dichlorophenol was previously investigated by Ensley et al. (1994) who found  $\text{EC}_{50}$  values of  $6.5 \text{ mg L}^{-1}$  in *Lemna gibba*.

As conventional wastewater treatment processes are unable to act as a reliable barrier concerning triclosan, it is discussed to introduce additional advanced treatment technologies in the areas where a pollution problem concerning triclosan and other persistent organic pollutants has been recognized or is anticipated. Ikehata et al. (2008) and Ternes et al. (2003) have evaluated different technologies including ozonation and advanced oxidation processes, membrane bioreactors, membrane filtration and activated carbon adsorption, suggesting that chemical oxidations using ozone is a highly effective treatment process for a wide spectrum of emerging organic pollutants, including pesticides, pharmaceuticals, personal care products, surfactants, microbial toxins and natural fatty acids. Ozone ( $\text{O}_3$ ) is a very powerful disinfecting and deodorizing gas. The ability of ozone to disinfect polluted water was recognized in 1886 by de Meritens (Vosmaer, 1916). However, the widespread introduction of ozone to remove pollution from drinking water started in the 1960s (Langlais et al., 1991). Nowadays, ozone is used in removing bacteria, viruses, algae and fungi as well as sulfur, thus also eliminating taste and odor problems, as well as oxidizing and mineralizing organic chemicals concerning drinking water (Langlais et al., 1991).

Ozonation has recently emerged as an important technology for the oxidation and destruction of a wide range of organic pollutants in wastewater as well (Ikehata et al., 2006). It has been proven to be an effective post-treatment technique for pharmaceuticals and personal care products (Carballa et al., 2007; Ikehata et al., 2008; Lee and von Gunten, 2010; Wert et al., 2009; Snyder et al., 2006).

Although ozonation of organic pollutants in wastewater has been investigated in numerous studies, data on the removal of triclosan and eventual formation of by-products are scarce and incomplete and they are mainly focused on the effect and kinetics of triclosan oxidation by aqueous ozone. Suarez et al. (2007) investigated that nearly 100% of triclosan depletion was achieved for a  $4 \text{ mg L}^{-1}$   $\text{O}_3$  dose applied to a wastewater containing  $7.5 \text{ mg L}^{-1}$  of DOC, while Wert et al.

(2009) reported that >95% triclosan removal was independent of water quality when the  $\text{O}_3$  exposure was measurable ( $0\text{--}0.8 \text{ mg min L}^{-1}$ ). However, Levy et al. (1999) investigated that the antibacterial activity of triclosan is derived primarily from its phenol ring, via van der Waals and hydrogen-bonding interactions with the bacterial enoyl-acyl carrier protein reductase enzyme. The characterization of the reaction pathways of the ozonation of triclosan is currently rather unclear. Furthermore, it is essential to understand all possible transformation products to enable a full risk assessment especially considering toxicological classification.

The objective of this work was to study the reaction products of triclosan formed during ozonation treatment. Additionally, the toxicity of selected by-products was investigated to evaluate the oxidative post-treatment technique with ozone in removal of triclosan in water and wastewater systems.

## 2. Materials and methods

### 2.1. Standards and reagents

Triclosan was purchased from Ehrenstorfer (Augsburg, Germany) with purity being  $\geq 99\%$  according to the supplier. Methanol, Toluene, Acetone, Methyl-*tert*-butyl ether (MTBE) were used in residue grade quality and purchased from Merck, Darmstadt, Germany. Triclosan stock solutions were prepared at a concentration of  $10 \text{ mg L}^{-1}$  according to its water solubility by dissolution of the solid compound in water (HPLC grade, Baker, Deventer, The Netherlands).

2,4-dichlorophenol, 4-chlororesorcinol, and 4-chlorocatechol were purchased from Sigma Aldrich.

$\text{O}_3$  stock solutions were prepared by purging an  $\text{O}_3$ -containing gas stream through HPLC water. The  $\text{O}_3$ -containing gas stream was produced by passing air through an  $\text{O}_3$  generator (Enaly 1000BT-12, Enaly M&E Ltd, Shanghai, China) at constant flow rate of  $0.5 \text{ L min}^{-1}$ . According to UV–Vis spectrophotometry (Shimadzu, Duisburg, Germany), the concentration of  $\text{O}_3$  stock solution was  $2 \text{ mg L}^{-1}$ .

In the toxicity tests the Chinese hamster ovary cells (CHO-9) were used and MTT (Sigma, St. Louis, USA) was used in the cytotoxicity test.

### 2.2. Ozonation and extractions

Samples were prepared by mixing  $\text{O}_3$  stock solutions into triclosan stock solutions in different volume ratios to reach the molar ration of triclosan: $\text{O}_3$  in 1:1, 1:3 and 1:5. All samples were extracted after the reaction performed at room temperature overnight. The pH of the water used was  $7 \pm 0.5$ . It was measured at the beginning of the experiments as well as at the end.

Samples were extracted by solid phase extraction (SPE) using polymeric cartridges (Strata-X, Phenomenex, Aschaffenburg Germany). Before the extraction, the SPE cartridges were rinsed with 6 mL methanol and 6 mL HPLC-grade water. After loading the samples to the cartridges, they were eluted by methyl-*tert*-butyl ether (MTBE) for analysis by gas chromatography-mass spectrometry (GC-MS), whereas duplicate samples were eluted by methanol for analysis by

high performance liquid chromatography-mass spectrometry (HPLC-MS/MS).

### 2.3. Analytical methods

Samples eluted by MTBE were analyzed by gas chromatography with mass spectrometric detection (GC-MS) equipped with a programmable temperature vaporizer (PTV) injector. The PTV (1  $\mu$ L injection volume) was operated in PTV splitless mode. The injection temperature of 115 °C was held for 3 s, it was successively ramped with 12 °C s<sup>-1</sup> to 280 °C for the transfer of the analytes. This temperature was held for 1.3 min. The injector was then ramped with 1 °C s<sup>-1</sup> to 300 °C which was held for 7 min as a cleaning phase.

The GC separation was performed with a DB-5MS column (J&W Scientific, Santa Clara, United States), L: 15 m; ID: 0.25 mm; film: 0.25  $\mu$ m using a temperature programme of: 100 °C (hold: 1 min) ramped with 5 °C min<sup>-1</sup> to 220 °C and with 30 °C min<sup>-1</sup> successively to 280 °C. Finally, the baking temperature 280 °C was held for 7 min. Helium (5.0) was used as a carrier gas with a flow rate of 1.3 mL min<sup>-1</sup>. The transfer line was held at 250 °C, which is sufficient to transfer all compounds from the GC into the MS as the vacuum builds up in the transfer line. The ion source of the mass spectrometer (DSQ, Thermo Finnigan, Dreieich, Germany) was operated at 230 °C in electron impact mode. The MS was used in full scan mode from 50 Da to 600 Da and the detector was operated with 1218 V.

The samples eluted by methanol were analyzed by liquid chromatography with tandem mass spectrometric detection (HPLC-MS/MS). The separation was performed using a Phenomenex synergi 4u polar-RP column (150  $\times$  2 mm I.D., particle size 4  $\mu$ m). The flow rate was 0.25 mL min<sup>-1</sup>. The LC gradient was established by mixing two mobile phases: phase A, HPLC water and phase B, Methanol. The chromatographic separation was achieved with the following gradient: 0–2 min 100% A, changing to 100% B in 30 min, 32–36 min 100% B. The injection volume was 10  $\mu$ L.

The LC system consisted of a UltiMate 3000 autosampler (WPS-3000 T SL), a UltiMate 3000 pump (DG-3600 M), a UltiMate 3000 column compartment hold (TCC-3000 RS) on 20 °C (all from Dionex, California, United States). After LC separation, the analytes were determined by an AB-Sciex (California, United States) API 4000 triple quadrupole mass spectrometer using electrospray ionization in negative mode with full scan from 130 Da to 400 Da utilizing the primary quadrupole. Nitrogen was used as a drying (at 400 °C) nebulizing and collision gas. One scan per second was recorded.

### 2.4. Toxicity tests

#### 2.4.1. Cell culture

The Chinese hamster ovary cells (CHO-9) were cultured in HAM's F12 medium supplemented with 10% Foetal Calf Serum, 0.5% gentamycin and 0.5% L-glutamine at 37 °C and 5% CO<sub>2</sub> conditions.

#### 2.4.2. Exposure

Triclosan and 2,4-dichlorophenol were tested between 0.5 and 100  $\mu$ g L<sup>-1</sup> for 24 h.

#### 2.4.3. Cytotoxicity: MTT test

To detect cytotoxic effects the MTT test was performed using the 96-well plate format using 100,000 CHO-9 cells in 200  $\mu$ L of HAM's F12 medium in each well. After 24 h the fresh medium was added and the cells were exposed to the different concentrations of the two substances for another 24 h. After the exposure time the medium was removed and 100  $\mu$ L fresh medium and 10  $\mu$ L MTT solution (5 mg MTT dissolved in 1 mL phosphate buffered saline) (KCl 2.67 mM, KH<sub>2</sub>PO<sub>4</sub> 1.47 mM, NaCl 137.93 mM, Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O 8.06 mM; Invitrogen) were added to each well and incubated at 37 °C for 2 h. The medium was then replaced with 100  $\mu$ L of lysis solution (99.4 mL dimethylsulfoxide, 0.6 mL acetic acid [100%] and 10 g sodium dodecyl sulfate) and the absorption was directly measured at 590 nm.

#### 2.4.4. Genotoxicity: alkaline comet assay

The alkaline comet assay was performed as described by Ostling and Johanson (1984) and later on revised by Singh et al. (1988) with some minor modifications. In short: Microgels were prepared by adding 50  $\mu$ L of low melting point agarose (L.M.P. agarose) to a chamber. 100,000 CHO-9 cells were exposed to different concentrations of triclosan and 2,4-dichlorophenol for 24 h. 0.1 mg L<sup>-1</sup> N-ethyl-N-nitrosourea was used as a positive control and added to the cells 30 min prior to trypsinization. After the exposure time the cells were washed, trypsinized and resuspended. 45  $\mu$ L of low melting point agarose were mixed with 20  $\mu$ L of cell suspension containing 8000 cells and added on top of the first layer of agarose. After solidification the slides were covered with freshly prepared and precooled lysis solution overnight at 4 °C. Before electrophoresis the slides were incubated in electrophoresis solution at 4 °C for 20 min. Electrophoresis was then performed for 20 min at 4 °C with 300 mA. After electrophoresis the slides were incubated in neutralization solution for 30 min and afterward dehydrated in ethanol for 2 h. Then the slides were stored overnight to let the gels dry completely. DNA was stained with SYBR Green® and image analysis was performed using the Comet Assay IV Software (Perceptive Instrument, UK) and a CCD (charge coupled device) camera attached to a Leica microscope. All experiments were carried out three times and statistical analysis was performed using the Mann–Whitney test.

## 3. Results and discussions

### 3.1. Identification of triclosan ozonation products

In Table 1 it can be seen that triclosan reacts under all used conditions quantitatively with ozone, thus removal rates of 94–99.9% seem realistic.

However, four major peaks were detected in the gas chromatogram of a sample extract of ozonized triclosan sample with molar ratio of triclosan:ozone in 1:2 measured by GC-MS. On the basis of their mass spectra, isomers of dichlorophenol (M1) and chlorocatechol (M2) were identified at the retention time of 5.88 min and 12.7 min, respectively. Peaks at retention time of 24.99 min and 28.94 min were identified as triclosan and its mono-hydroxylated product (M3). By comparing to a true standard, 2,4-dichlorophenol was verified as the major monoaromatic metabolite in GC-MS. The full results of all

**Table 1 – Volumes, molar ratios, and initial concentrations of triclosan and ozone in samples in comparison to final triclosan concentrations and removal rates.**

Sample Name	Volume of triclosan stock solution [mL]	Volume of aqueous ozone [mL]	Molar ratio of triclosan to ozone	Initial concentration		Residual triclosan [mg L <sup>-1</sup> ]	Removal rate [%]
				Triclosan [mg L <sup>-1</sup> ]	Ozone [mg L <sup>-1</sup> ]		
Sample 1	45	55	1:1	4.5	1.1	0.26	94
Sample 2	29	71	1:3	2.9	1.42	0.087	97
Sample 3	14	86	1:5	1.4	1.72	0.001	99.9

transformation products identified by GC-MS are listed in Table 2.

A chromatogram of a sample with the molar ratio of triclosan:ozone: 1:2 measured by HPLC-MS is shown in Fig. 1. Similar to the results from GC-MS, dichlorophenol (M1), chlorocatechol (M2) and mono-hydroxy-triclosan (M3) were identified in the chromatogram. They were detected in different ratios by GC-MS and HPLC-MS because of different extraction and detection methods. Additionally, two isomers of chlorocatechol (M2) and di-hydroxy-triclosan (M4) were detected by the HPLC-MS measurements. The dichlorophenol (M1) was confirmed as 2,4-dichlorophenol by comparison to a standard

purchased from Sigma Aldrich (Steinheim, Germany), the two isomers of M2 were confirmed as 4-chlorocatechol (4-chloro-1,2-dihydroxybenzene) (M2a) and 4-chlororesorcinol (4-chloro-1,3-dihydroxybenzene) (M2b) by comparison with standards purchased from Sigma Aldrich (Steinheim, Germany).

Other transformation products did not comply with standards by means of the retention time or were not commercially available. Therefore, collision-induced dissociation (CID) was used to produce product ion scans for further metabolite identification. For this purpose the [M-H]<sup>-</sup> ion was selected as precursor ion. The HPLC-MS/MS results of the metabolite identification are listed in Table 3.

**Table 2 – GC-MS results of the transformation product identification.**

Compound	Structure	Retention time [min]	MW [Da]	RT + MS complied standard	RT + MS complied theory	Mass fragments (including the Cl isotope signals) [Da]
Triclosan (M)		24.99	288	Yes	Yes	288 (290, 292), 252 (254, 256), 218 (220)
2,4-Dichlorophenol (M1)		5.88	162	Yes	Yes	162 (164, 166), 126, 98, 63
Chlorocatechol (M2)		11.40	144		Yes	144 (146), 115, 81, 52
Mono-hydroxy-triclosan (M3)		28.45	304		Yes	304 (306, 308), 234 (236, 238)

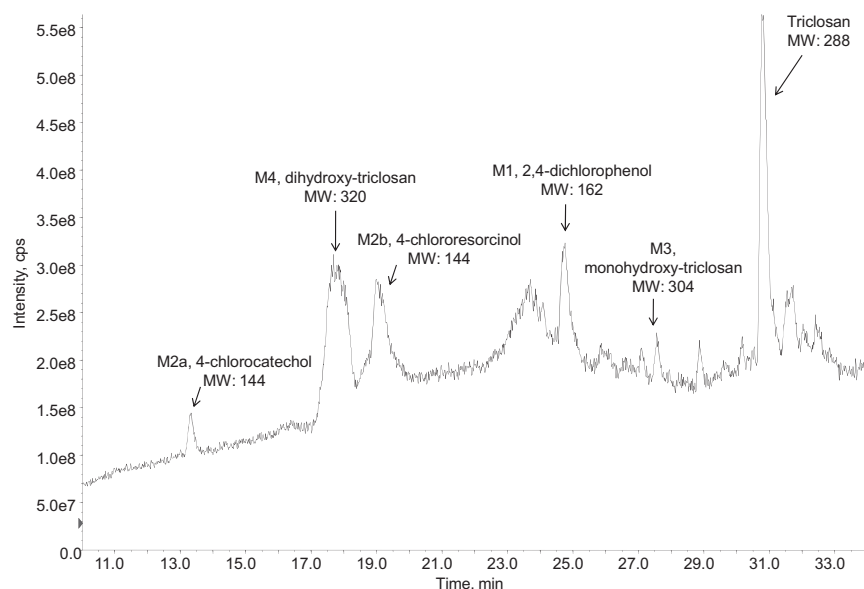


Fig. 1 – Chromatogram of sample with triclosan:ozone in 1:2 from HPLC-MS/MS (Electrospray ionization in negative polarization ESI (–)) with suggested identification.

Mono-hydroxy-derivatives of triclosan (M3) were detected at 303 and 305 Da (equivalent to the two main isotope signal for the  $(M-H)^-$  ion) at 27.56 min retention time. The product ion scan of 303 Da and 305 Da provided abundant fragmentation for this compound (Fig. 2). The identification was confirmed by the detection of the fragment ion peak at 161 corresponding to  $(C_6H_2OCl_2)^-$ . The two chlorine atoms are being verified by the chlorine isotope distribution in Fig. 2C. Further analysis of Fig. 2B and C shows that fragments 125 Da and 113 Da are attributed to  $[C_6H_2OCl]^-$  and  $[C_6H_2OCl]^-$  stemming from cleavage of HCl and CHCl from 161 respectively. It can thus be hypothesized that the oxidation takes place in the triclosan ring with less chlorination.

The molecular ion peak of di-hydroxy-triclosan (M4) was detected with the retention time at 17.65 min (Fig. 1). The product ion spectrum of M4 showed major fragment ion peaks at 161 and 125 Da, indicating that the double chlorinated ring is again still intact and not oxidized (Fig. S1). Similar as the fragmentation spectrum of M3, the transformation product identification was further confirmed by an investigation on the chlorine isotope peaks.

### 3.2. Structural suggestions and verifications

After triclosan was reacted with ozone, some intermediates were identified by using GC-MS and HPLC-MS/MS. On the basis of their GC-MS spectra and HPLC-MS/MS fragmentation, several ozonation products for triclosan are proposed (Table 2

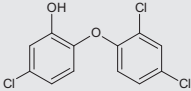
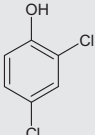
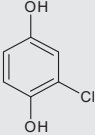
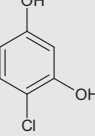
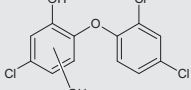
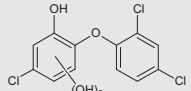
and 3). Triclosan can be oxidized by ozone resulting in OH addition forming mono-hydroxy- (M1) and di-hydroxy-triclosan (M2) and finally breaking of the ether bond resulting in 2,4-dichlorophenol (M1), 4-chlorocatechol (M2a) and 4-chlororesorcinol (M2b).

The 2,4-dichlorophenol (M1) is a well known product of triclosan which has been detected by several investigators within biodegradation experiments (Kim et al., 2010), as an oxidative transformation product from reactions with manganese oxides (Zhang and Huang, 2003), as well as a photochemical degradation product in both natural and buffered deionized water (Latch et al., 2005). Kim et al. (2010) has found the chlorocatechol (M2), mono-hydroxy-triclosan (M3) and di-hydroxy-triclosan (M4) as biodegradation products of triclosan from bacteria. Additionally, Zhang and Huang (2003) have detected that mono-hydroxy-triclosan (M3) could be one of the oxidation products of triclosan by manganese oxides. Except the 2,4-dichlorophenol (M1), none of the other transformation products have been published as ozonation by-products of triclosan, to the best of our knowledge.

### 3.3. Ozonation of triclosan

The triclosan chromatograms of the three samples from the experiment are shown in Fig. 3. Complete ozonation of triclosan (but not its transformation products) was detected in the sample with molar ratio of triclosan:ozone = 1:5. Ozonation was substantial in the sample with a molar ratio of

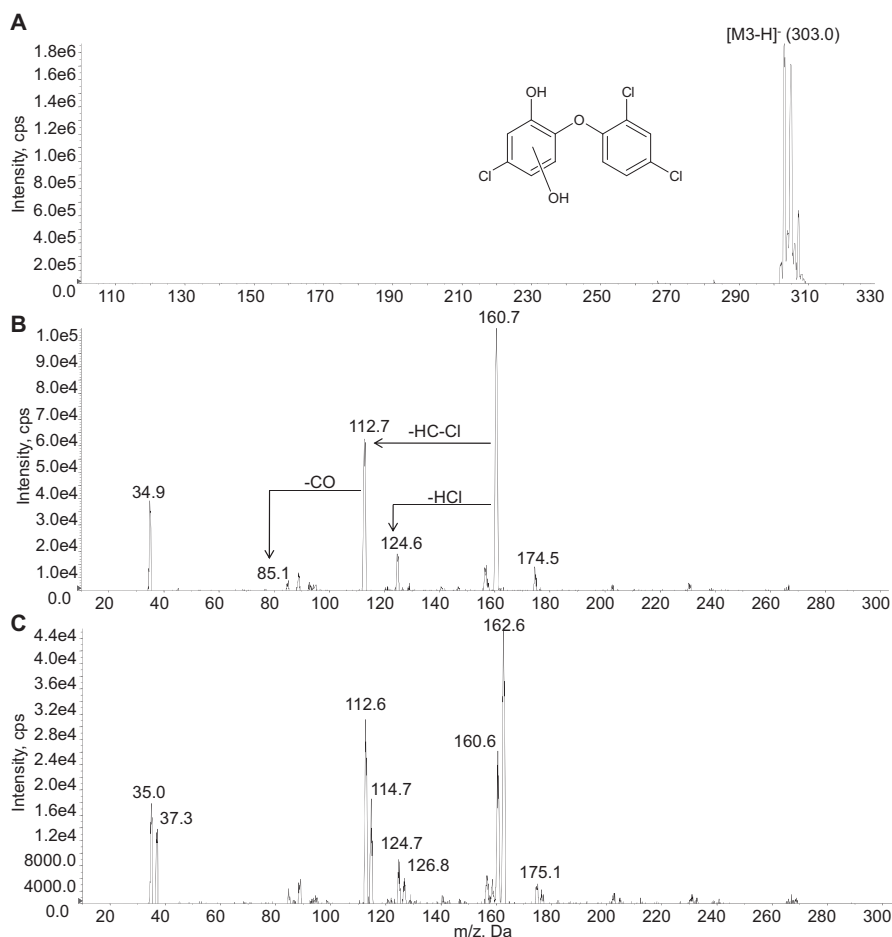
**Table 3 – HPLC-MS/MS results of the transformation product identification.**

Compound	Structure	Retention time [min]	MW [Da]	RT + MS complied standard	RT + MS complied theory	Product ion scan fragments [Da]
Triclosan (M)		30.78	288	Yes	Yes	287, 252, 251
2,4-Dichlorophenol (M1)		24.76	162	Yes	Yes	161, 125, 89, 61, 35
4-Chlorocatechol (M2a)		13.31	144	Yes	Yes	143, 107, 79, 51, 35
4-Chlororesorcinol (M2b)		19.19	144	Yes	Yes	143, 107, 79, 51, 35
Mono-hydroxy-triclosan (M3)		27.56	304		Yes	303, 161, 125, 113, 85, 35
Di-hydroxy-triclosan (M4)		17.65	320		Yes	319, 161, 125, 35

triclosan:ozone = 1:3 and less in the sample with molar ratio of triclosan:ozone = 1:1.

2,4-dichlorophenol was detected by GC-MS and HPLC-MS in all the three samples. Levels of 2,4-dichlorophenol in the

sample with high ozone amount (molar ration of triclosan:ozone in 1:3) were, however, lower than the other two samples with lower ozone amount, which indicate that 2,4-dichlorophenol is an intermediate product and can be



**Fig. 2** – HPLC-MS/MS analysis (ESI<sup>-</sup>) of mono-hydroxy-triclosan, **A**) the full scan spectrum of the mono-hydroxy-triclosan, **B**) product ion spectrum of the precursor 303 of mono-hydroxy-triclosan (the isotopic composition <sup>35</sup>Cl<sub>3</sub>), **C**) product ion spectrum of the precursor of mono-hydroxy-triclosan (the isotopic composition <sup>35</sup>Cl<sub>2</sub> <sup>37</sup>Cl).

further oxidized. Chromatograms of 2,4-dichlorophenol and triclosan during ozonation showing the somewhat longer presence of 2,4-dichlorophenol are shown in Fig. 3. The conversion yields of triclosan to 2,4-dichlorophenol depend on the amount of ozone. Now, these compounds are available they can be studied in biodegradation processes as well. More information can be gained from Fig. S2 in which the signal height obtained by HPLC-ESI<sup>-</sup> MS of all identified transformation products is plotted against the relative ozone concentration.

#### 3.4. Toxicity of triclosan transformation products in comparison to triclosan

Cytotoxic and genotoxic effects of triclosan and its oxidation by-product 2,4-dichlorophenol were analyzed using the MTT test and the alkaline comet assay.

These two tests are well established toxicity tests and have been used in the testing of chemicals for several decades and they have been proven to be rapid and sensitive methods. The MTT tests give a quantitative measure on the amount of



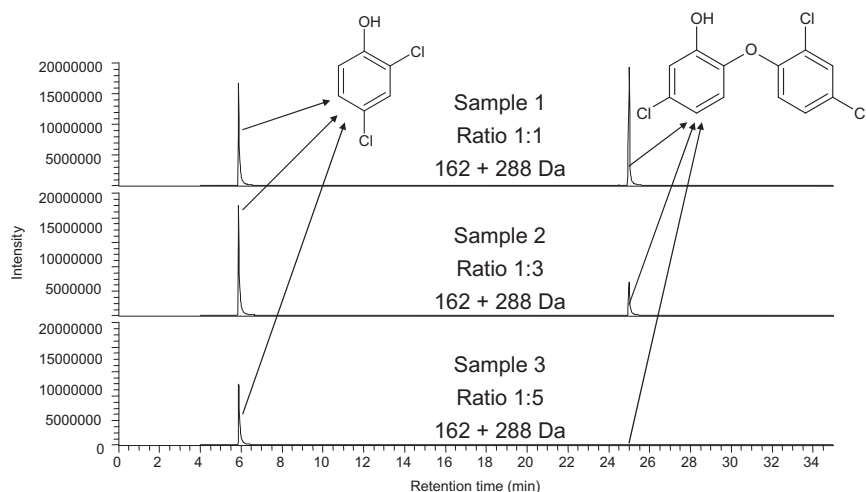


Fig. 3 – Chromatogram and MS spectra of triclosan and its ozonation product – 2,4-dichlorophenol detected by GC-MS in electron impact ionization. To simplify the graph, the signals for 162 and 288 Da were added to gain one chromatogram for both 2,4-dichlorophenol (162 Da) and triclosan (288 Da). Sample 1, triclosan:ozone = 1:1. Sample 2, triclosan:ozone = 1:3. Sample 3, triclosan:ozone = 1:5.

viable and dead cells thus the cytotoxicity of the tested substances resulting in an idea about the general toxicity (Mosmann, 1983). DNA damage was measured by the alkaline comet assay which allows the detection of single and double strand breaks as well as alkali labile sites (Singh et al., 1988;

Tice et al., 2000). In addition both tests have been previously adapted for the use of CHO (Chinese Hamster Ovary) cells. This is a cell line which has been derived from the ovaries of the Chinese hamster in 1957 and widely used in toxicity testing (Puck et al., 1958).

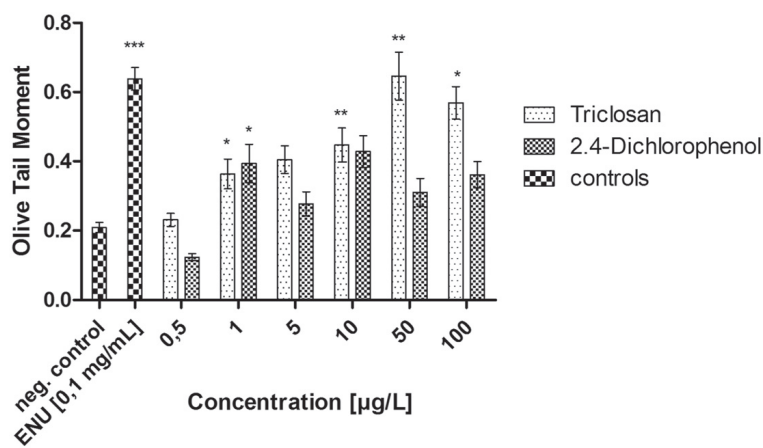


Fig. 4 – Genotoxic effects of triclosan and 2,4-dichlorophenol on CHO-9 cells after 24 h of exposure investigated using the alkaline comet assay. Asterisks display the significance in DNA damage increase ( $p < 0.05$  = \* significant;  $p < 0.01$  = \*\* very significant;  $p < 0.001$  = \*\*\* extremely significant).

Both substances were tested at concentrations between 0 and 100  $\mu\text{g L}^{-1}$ . The results of the MTT test show that neither triclosan nor 2,4-dichlorophenol has any cytotoxic effect on CHO-9 (Chinese Hamster Ovary) cells after a 24 h exposure at the used concentrations (data not shown). For each concentration the viability lies above 90% compared to the negative control. In addition no difference can be seen comparing both substances and their effects on cell viability.

However the results of the alkaline comet assay with triclosan show tail moments increasing with concentrations. Compared to triclosan and the negative control, 2,4-dichlorophenol is less genotoxic (Fig. 4). This indicates that ozonation is a useful tool in removing the genotoxic compound triclosan from wastewater. However, it should be taken into account that 2,4-dichlorophenol is prioritized under the EU surface water directive 76/464/EC (European Commission 1976) (Umweltbundesamt, 2005) and is classified to be “harmful to aquatic organisms” and “may cause long-term adverse effects in the aquatic environment”.

#### 4. Conclusion

Contamination of surface water and ground water with triclosan is an emerging issue in environmental science and engineering. The outcomes of this study are:

- Removal of triclosan from water can be achieved by using ozonation.
- The treatment process can eliminate triclosan completely and convert it into the products: 2,4-dichlorophenol, chlorocatechol, mono-hydroxy-triclosan and di-hydroxy-triclosan. Increasing the ozone concentrations in the reactions leads to decreased concentration of triclosan as well as its oxidation by-products.
- 2,4-dichlorophenol shows lower genotoxic effects than triclosan at the tested concentrations, but this compound is classified to be toxic to aquatic organisms. The other transformation products cannot be assessed up to now.
- Formation and occurrence of the identified transformation products should be investigated at full scale applications
- Reactor design should take the formation of oxidation by-products into account and possibly use higher ozone doses or retention times to remove by-products.

#### Acknowledgment

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#### Appendix. Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.watres.2012.01.039.

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### **Research paper 7:**

Lolas IB., **Chen XJ.**, Bester K. and Nielsen JL. Identification of triclosan degrading bacteria using stable isotope probing and microautoradiography. Microbiology 158 (2012) 2805-2814



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## Identification of triclosan-degrading bacteria using stable isotope probing, fluorescence *in situ* hybridization and microautoradiography

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Triclosan is considered a ubiquitous pollutant and can be detected in a wide range of environmental samples. Triclosan removal by wastewater treatment plants has been largely attributed to biodegradation processes; however, very little is known about the micro-organisms involved. In this study, DNA-based stable isotope probing (DNA-SIP) combined with microautoradiography-fluorescence *in situ* hybridization (MAR-FISH) was applied to identify active triclosan degraders in an enrichment culture inoculated with activated sludge. Clone library sequences of 16S rRNA genes derived from the heavy DNA fractions of enrichment culture incubated with <sup>13</sup>C-labelled triclosan showed a predominant enrichment of a single bacterial clade most closely related to the betaproteobacterial genus *Methylobacillus*. To verify that members of the genus *Methylobacillus* were actively utilizing triclosan, a specific probe targeting the *Methylobacillus* group was designed and applied to the enrichment culture incubated with <sup>14</sup>C-labelled triclosan for MAR-FISH. The MAR-FISH results confirmed a positive uptake of carbon from <sup>14</sup>C-labelled triclosan by the *Methylobacillus*. The high representation of *Methylobacillus* in the <sup>13</sup>C-labelled DNA clone library and its observed utilization of <sup>14</sup>C-labelled triclosan by MAR-FISH reveal that these micro-organisms are the primary consumers of triclosan in the enrichment culture. The results from this study show that the combination of SIP and MAR-FISH can shed light on the networks of uncultured micro-organisms involved in degradation of organic micro-pollutants.

## INTRODUCTION

Triclosan [5-chloro-2-(2,4-dichloro-phenoxy)-phenol] is a synthetic antibacterial compound that inhibits the NADH-dependent enoyl-[acyl-carrier protein] reductase, an essential enzyme involved in the biosynthesis of fatty acids (Heath *et al.*, 1999; McMurry *et al.*, 1998; Regös *et al.*, 1979). As an effective antimicrobial agent, triclosan has been used in a wide range of personal care products, such as toothpaste and soaps, and in consumer products, including textile and plastics (DeSalva *et al.*, 1989; Jones *et al.*, 2000; Schweizer, 2001). Due to its extensive use and persistence, triclosan and some of its derivatives can be

detected in different environmental matrices such as wastewaters, surface waters and sediments, and in biological samples, including those from fish, algae, human plasma, urine and breast milk (Balmer *et al.*, 2004; Hovander *et al.*, 2002; Miller *et al.*, 2008; Sánchez-Brunete *et al.*, 2010; Sandborgh-Englund *et al.*, 2006; Wilson *et al.*, 2003; Ye *et al.*, 2008).

Biodegradation of triclosan has been shown by mixed bacterial cultures from activated sludge (Gangadharan Puthiya Veetil *et al.*, 2012; Hay *et al.*, 2001; Stasinakis *et al.*, 2010) and in wastewater treatment plants (WWTPs) (Bester, 2003; Chen *et al.*, 2011; Singer *et al.*, 2002). Due to insufficient removal during wastewater treatment, triclosan has been found in WWTP effluents in concentrations ranging from 1 to 10 µg l<sup>-1</sup> (Adolfsson-Erici *et al.*, 2002; Bester, 2003, 2005; Lindström *et al.*, 2002; Singer *et al.*, 2002). Although mass balance assessments have shown that biological treatment contributes to the major removal of triclosan in WWTPs (Bester, 2003; Heidler & Halden, 2007; Singer *et al.*, 2002), little is known about the

**Abbreviations:** DGGE, denaturing gradient gel electrophoresis; FISH, fluorescence *in situ* hybridization; MAR, microautoradiography; SIP, stable isotope probing; WWTP, wastewater treatment plant.

The GenBank/EMBL/DDJB accession numbers for the sequences of the triclosan-degrading culture clones represented in the phylogenetic tree are JX099503–JX099536.

A supplementary figure is available with the online version of this paper.

actual mechanisms or the micro-organisms involved in the degradation process. So far, two wastewater isolates, *Sphingomonas* sp. strains Rd1 (Hay *et al.*, 2001) and PH-07 (Kim *et al.*, 2011), have been shown to degrade triclosan via co-metabolism. Meade *et al.* (2001) showed that two soil bacteria, *Pseudomonas putida* and *Alcaligenes xylosoxidans*, have a high resistance to triclosan and can utilize it as their sole carbon source, and the nitrifying *Nitrosomonas europaea* has also been shown to biodegrade triclosan (Roh *et al.*, 2009). Recently, several triclosan-degrading strains belonging to the genus *Pseudomonas* were isolated from aerobic and anaerobic enrichment cultures of activated sludge (Gangadharan Puthiya Veetil *et al.*, 2012). Biodegradation of triclosan has also been reported in fungi (Hundt *et al.*, 2000). However, knowledge based on culture-independent approaches of the identity and ecophysiology of triclosan-degrading bacteria in complex microbial systems is still limited.

Stable-isotope probing (SIP) allows for *in situ* detection of bacterial communities capable of metabolizing a specific carbon source and thus links function to identity without the need to culture the bacteria involved (Radajewski *et al.*, 2000). SIP approaches have been used to identify various types of environmental pollutant-degrading bacteria, e.g. those able to degrade nonylphenols (Zemb *et al.*, 2012), toluene (Woods *et al.*, 2011) and phenols (Manefield *et al.*, 2007).

The aim of this study was to use the SIP approach to identify the active micro-organisms in a triclosan-degrading consortium derived from activated sludge exposed to  $^{13}\text{C}$ -labelled triclosan. Genomic fingerprinting analyses of resolved  $^{13}\text{C}$ -labelled DNA allowed the design of a specific fluorescence *in situ* hybridization (FISH) probe for a putative triclosan-degrading phylotype, which was used in combination with microautoradiography (MAR) to verify the physiology and abundance of these micro-organisms in the enrichment.

## METHODS

**Reagents and media.** Triclosan (Irgasan) was purchased from Sigma-Aldrich with a purity of >97%.  $^{13}\text{C}_{12}$ -Labelled triclosan (isotope purity >98%) was purchased from Wellington Laboratories and was dissolved in methanol. [Dichlorophenyl- $\text{U-}^{14}\text{C}$ ]-labelled triclosan (specific activity  $5.43 \text{ MBq mg}^{-1}$ ) was donated by Ciba. Stock solutions of both radiolabelled and unlabelled triclosan were prepared in acetone. As a standard procedure, substrate solutions were allowed to dry at room temperature prior to the addition of specified media or culture. For all experiments, nitrate mineral salts medium (NMS) was used as carbon-free medium (Whittenbury *et al.*, 1970). All other reagents used for SIP and MAR were commercial products of highest grade (Chen *et al.*, 2011; Kristiansen *et al.*, 2011a; Neufeld *et al.*, 2007).

**Enrichment culture and growth conditions.** Activated sludge was taken from the aeration tanks from C/N/P-removing Aalborg West WWTP (Aalborg, Denmark) and was used as source of inoculum for enrichment of triclosan-degrading organisms. The initial incubation has been described in a preliminary report (Chen *et al.*, 2011). Briefly,

an activated sludge sample was spiked with  $2 \text{ mg triclosan l}^{-1}$  and incubated in the dark under aerobic conditions at  $22\text{--}25^\circ\text{C}$  on a rotary table (150 r.p.m.). Following the initial incubation and every 9 days thereafter, the enrichment culture was transferred [10% (v/v)] to fresh NMS medium containing  $2 \text{ mg triclosan l}^{-1}$ . The enriched culture had a maximum cell density of  $6 \times 10^8 \text{ cells ml}^{-1}$  and was maintained for 4 months before conducting the SIP and MAR incubations.

### Analytical methods

**Liquid–liquid extraction.** Samples (5 ml) from the experiments were extracted by addition of 2 ml toluene and  $100 \mu\text{l}$  internal standard solution ( $1000 \text{ ng musk xylene D}_{15} \text{ ml}^{-1}$ ) and were vigorously stirred for 5 min. The organic phase was extracted and the residual water was removed by freezing the samples overnight at  $-20^\circ\text{C}$ . These organic extracts were then concentrated to 1 ml with a nitrogen flow condenser at  $55^\circ\text{C}$ .

**Instrumental analysis.** Triclosan extracts were finally analysed by gas chromatography with mass spectrometric detection (GC-MS, Thermo-Trace-MS and Trace GC) equipped with a splitless injector and A200S autosampler. Samples ( $1 \mu\text{l}$ ) were injected into the injector in splitless (1.5 min) mode held at  $240^\circ\text{C}$ . The GC separation was performed with an Rxi-5Sil MS column (Restek): length, 10 m; ID, 0.18 mm; film, 0.18  $\mu\text{m}$ ; and a temperature programme of  $90^\circ\text{C}$  (hold 1 min) ramped at  $50^\circ\text{C min}^{-1}$  to  $135^\circ\text{C}$  and then at  $10^\circ\text{C min}^{-1}$  to  $220^\circ\text{C}$ . Finally, the baking temperature was reached by ramping the column at  $40^\circ\text{C min}^{-1}$  to  $260^\circ\text{C}$  which was held for 6 min. Helium (5.0) was used as carrier gas with a flow rate of  $1.3 \text{ ml min}^{-1}$ . The transfer line of the mass spectrometer (Trace MS, Thermo Finnigan) was held at  $250^\circ\text{C}$ . The ion source was operated at  $160^\circ\text{C}$ . The mass spectrometer was operated in selected ion mode (SIM) utilizing 31–61 ms dwell time. The detector of the mass spectrometer was operated at 450 V. The recovery rate of triclosan was  $88 \pm 11\%$  (SD) and limit of quantification was  $3 \text{ ng g}^{-1}$ , as reported by Bester (2003).

**SIP.** A total of 5 ml (approx. 100 mg dry matter) of the enriched culture was transferred to 60 ml serum bottles and incubated with  $2 \text{ mg }^{13}\text{C}$ -labelled triclosan  $\text{l}^{-1}$  for 3 days. Parallel incubations were also prepared with unlabelled substrate and used as controls for verification of DNA-SIP labelling and triclosan degradation. The bottles were crimp-sealed with rubber stoppers and incubated in the dark at  $24^\circ\text{C}$  on a rotary table (150 r.p.m.) for 3 days. Subsequently, total DNA was extracted using the FastDNA SPIN kit for Soil (MP Biomedicals) according to the manufacturer's instructions. The DNA concentration was measured on a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). All incubations were carried out as biological duplicates.

**Isolation and fingerprinting of  $^{13}\text{C}$ -labelled DNA.** Caesium chloride ( $\text{CsCl}$ ) gradient fractionation, DNA precipitation and DNA quantification were set up as described previously (Neufeld *et al.*, 2007). Briefly,  $2 \mu\text{g}$  DNA from each sample (two control samples and two  $^{13}\text{C}$ -labelled samples) was added to the gradient buffer and mixed with  $\text{CsCl}$  to a final density of  $1.725 \text{ g ml}^{-1}$ . These solutions were added to 5.1 ml polyallomer Quik-seal centrifuge tubes (Beckman Coulter) and ultracentrifuged at  $133\,000 \text{ g}_{\text{av}}$  for 72 h at  $20^\circ\text{C}$  in a Sorvall TH-641 swing-out rotor (Kendro). Immediately after centrifugation, the density gradients were fractionated into 12 volumes of approximately  $400 \mu\text{l}$ . The buoyant density of each fraction was determined by measuring  $5 \mu\text{l}$  from each sample on a refractometer (AR200, Reichert). DNA from each fraction was precipitated with polyethylene glycol and glycogen as described elsewhere (Neufeld *et al.*, 2007), and followed by resuspension in

nuclease-free water. DNA was quantified using a NanoDrop 2000 spectrophotometer.

The shift in community between the control and the labelled fraction was visualized by molecular profiling using denaturing gradient gel electrophoresis (DGGE) and PCR. DGGE was performed as described in detail elsewhere (Kristiansen *et al.*, 2011a). From DGGE results, distinct DNA bands from the labelled heavy fractions (buoyant density 1.83 and 1.79 g ml<sup>-1</sup>) were chosen for subsequent sequencing (Fig. S1, available with the online version of this paper). Furthermore, the <sup>12</sup>C and <sup>13</sup>C-labelled DNA fractions were used as template for PCR with the 16S rRNA gene-targeted primers 26F/1492R (approx. 1450 bp product) (Lane, 1991). PCR conditions are described elsewhere (Kristiansen *et al.*, 2011b). A 16S rRNA clone library was prepared from the high density fractions (1.76–1.80 g ml<sup>-1</sup>) of the SIP incubation with the <sup>13</sup>C-labelled triclosan. The clone library preparation and the phylogenetic analysis were performed as described by Kristiansen *et al.* (2011a) except that the alignment and phylogenetic tree construction were done using MEGA 5 (Tamura *et al.*, 2011). Screening of the clone sequences with Bellerophon v3 (DeSantis *et al.*, 2006) did not identify any putative chimeras. Sequences represented in the phylogenetic tree were named triclosan-degrading culture clones and deposited in the GenBank database under accession numbers JX099503–JX099536.

**FISH probe design.** The 16S rRNA gene sequences from the clone library were used to design an oligonucleotide probe (Meth1138) (Table 1) using the probe design tool in the ARB software package (Ludwig *et al.*, 2004); the probe was subsequently confirmed for specificity using the CHECK PROBE programme in the Ribosomal Database Project (Maidak *et al.*, 2000). Optimum hybridization stringency for the probe was determined by performing formamide dissociation series on biomass from the enrichment culture and activated sludge from Aalborg West WWTP with 10% formamide (v/v) increments across a range of 0–60% (v/v). Prior to FISH, samples were homogenized and fixed with 4% (w/v) paraformaldehyde, as described previously (Nielsen, 2009). The group-specific probe Meth1138 was labelled with sulfoindocyanine dyes (Cy3). FISH analysis was performed by using the general bacterial probe mixture EUBmix labelled with 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS) and more specific probes labelled with Cy3 (Table 1). The FISH procedure was carried out as described previously (Nielsen, 2009). An epifluorescence microscope (Axioscope 2, Carl Zeiss) was used in all FISH analyses. Bacterial abundance was quantified by measuring the ratio of the area fluorescing with a probe (Cy3 labelled) to the area fluorescing with EUBmix probe (FLUOS labelled) on the same microscopic field. For each enumeration, at least 20 images were taken from two separate hybridizations and analysed using ImageJ software (Collins, 2007). FISH analyses were also conducted on samples taken from seven Danish WWTPs:

Bjergmarken, Aalborg East, Egå, Ejby, Hjørring, Skive and Aalborg West. These plants represent stable and well-functioning C/N/P-removing treatment plants with different configurations and influent wastewater composition.

**Microautoradiography.** Microautoradiography experiments in enrichment culture in combination with FISH (MAR-FISH) were performed as described previously (Nielsen & Nielsen, 2005). Briefly, 5 ml of the enriched culture was transferred to 9 ml serum bottles and incubated with 10 µCi <sup>14</sup>C-labelled triclosan (3.7 × 10<sup>7</sup> Bq) and unlabelled triclosan to a final concentration of 2 mg l<sup>-1</sup> under aerobic conditions for 1 day on a rotary table (labelled and unlabelled triclosan was added at time 0). As a control for chemography, a sample from the enriched culture was pasteurized at 70 °C for 10 min prior to MAR incubation and run in parallel. MAR incubations were terminated by fixing samples with 4% (w/v) paraformaldehyde. The samples were then washed, homogenized and immobilized on gelatin-coated coverslips as described elsewhere (Nielsen & Nielsen, 2005). Finally, the samples were subjected to FISH. After the FISH procedure, the samples were coated with liquid film emulsion (Kodak) and exposed in the dark for 3–6 days before being developed and microscopically examined. Production of <sup>14</sup>C-labelled CO<sub>2</sub> was monitored in MAR-incubated culture by measuring the percentage accumulation of precipitated radioactivity using a liquid scintillation counter (Packard 1600 TR; Packard) as follows. Samples (1 ml) from the headspace gas were withdrawn using a syringe and mixed with 1 ml 0.1 M NaOH solution in a gas-tight sealed serum bottle. At the same time, 0.1 ml aliquots were withdrawn from the culture and directly transferred to 3 ml scintillation liquid (Ultima Gold XR; Packard) to measure the total radioactivity of the culture. All incubations were carried out as biological duplicates.

## RESULTS

### Biodegradation of triclosan in enrichment culture

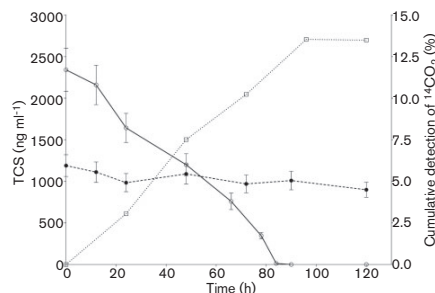
After spiking the enrichment culture with 2 mg triclosan l<sup>-1</sup>, the concentration of triclosan was reduced below the limit of quantification (3 ng l<sup>-1</sup>) within 90 h, whereas the triclosan concentration remained nearly constant in a pasteurized control spiked with 1 mg triclosan l<sup>-1</sup> (Fig. 1). This indicates that the removal of triclosan was predominantly due to biological activity, which agrees with the literature (Bester, 2005; Singer *et al.*, 2002). Degradation of 2 mg triclosan l<sup>-1</sup> followed first-order kinetics with a removal rate and half-life of 0.0431 h<sup>-1</sup> and 16 h, respectively. This was approximately five times faster than

**Table 1.** Oligonucleotides probes for FISH analysis

Probe	Specificity	Sequence (5'-3')	Reference or source
EUB338	Bacteria	GCT GCC TCC CGT AGG AGT	Amann <i>et al.</i> (1990)
EUB338II	Bacteria	GCA GCC ACC CGT AGG TGT	Daims <i>et al.</i> (1999)
EUB338III	Bacteria	GCT GCC ACC CGT AGG TGT	Daims <i>et al.</i> (1999)
Beta42a*	Betaproteobacteria	GCC TTC CCA CTT CGT TT	Manz <i>et al.</i> (1992)
Gam42a*	Gammaproteobacteria	GCC TTC CCA CAT CGT TT	Manz <i>et al.</i> (1992)
Meth1138*	<i>Methylobacillus</i> sp.	GCA CTC CAT GCT GCC GTT CG	This study
Meth1138-comp.	Competitor for Met1138	GCT CTC CAT GCT GCC GTT CG	This study

\*Used in equimolar concentrations with competitor.



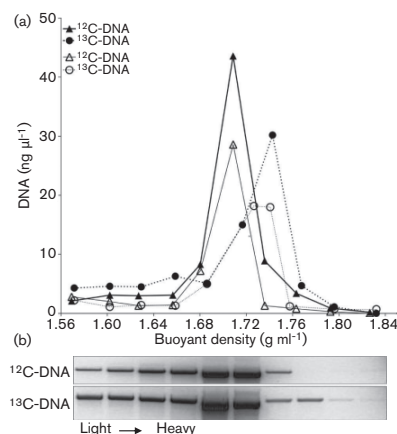


**Fig. 1.** Biodegradation of triclosan in the enrichment culture under aerobic conditions. The time-course of triclosan degradation ( $2 \text{ mg l}^{-1}$ ) in pasteurized ( $\bullet$ ) and active ( $\circ$ ) enriched culture is shown. Cumulative production of  $^{14}\text{C}$ -labelled  $\text{CO}_2$  ( $\square$ ) from the mineralization of  $^{14}\text{C}$ -labelled triclosan is shown on the secondary y-axis. Error bars for triclosan measurements indicate the 10% stated uncertainty from the method development by Bester (2003).

that calculated from degradation analyses where the same concentration of triclosan was spiked directly into activated sludge from the Aalborg West WWTP (Chen *et al.*, 2011). When radiolabelled triclosan was spiked into the enrichment culture, the subsequent liquid scintillation counts showed that this microbial community is able to mineralize triclosan, with approximately 13% of the added radioactivity detected in the headspace after 3 days of incubation (Fig. 1). The linear progression of  $^{14}\text{C}$ -labelled  $\text{CO}_2$  in the headspace indicates that triclosan degradation started immediately after its addition without a lag phase (Fig. 1). Meanwhile, no further accumulation of  $^{14}\text{C}$ -labelled  $\text{CO}_2$  was observed when the triclosan concentration was reduced below the detection limit.

#### Detection and phylogenetic analysis of $^{13}\text{C}$ -labelled bacterial 16S rRNA gene sequence

Following incubation with  $^{13}\text{C}$ -labelled triclosan, total DNA was extracted and centrifuged in a  $\text{CsCl}$  density gradient to separate labelled from non-labelled DNA. This resulted in a linear isopycnic gradient from 1.83 to  $1.57 \text{ g ml}^{-1}$  (Fig. 2). Although the buoyant densities in our SIP fractionation were relatively broad, a clear shift towards a heavier density of the quantified DNA was observed from both duplicate samples incubated with unlabelled triclosan relative to the  $^{12}\text{C}$ -labelled control. This shift was also apparent from the band intensity of the PCR product after 25 cycles of amplification of the 16S rRNA genes (Fig. 2). The shift in density was further evaluated by DGGE, revealing a shift in banding patterns in the heavy fractions (density  $1.80\text{--}1.76 \text{ g ml}^{-1}$ ) of the  $^{13}\text{C}$ -labelled triclosan incubation compared with the unlabelled control (Fig. S1). Distinct bands (1, 3, 6, 7, 8 and 9 on Fig. S1) were



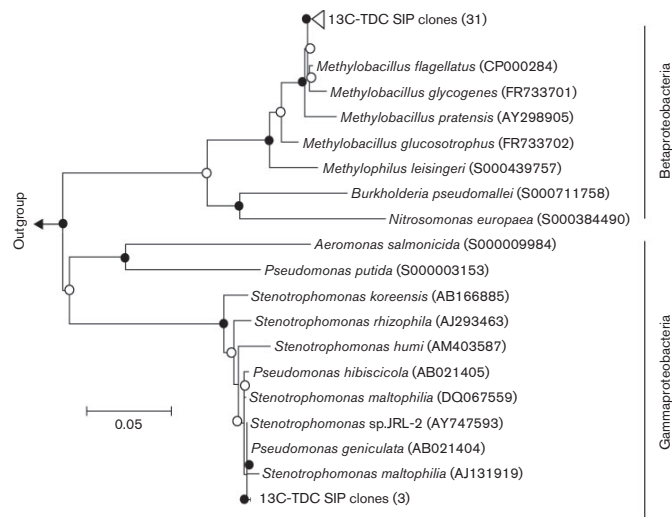
**Fig. 2.** Quantitative profiles of DNA in  $\text{CsCl}$  SIP gradients. The distribution of DNA in comparative SIP gradients of DNA from microcosms ( $n=2$ ) amended with  $^{12}\text{C}$ - or  $^{13}\text{C}$ -labelled triclosan is shown in (a). Open symbols show the results of the biological duplicate. (b) Band intensity of PCR products targeting the 16S rRNA genes ( $\sim 1.5 \text{ kb}$ ) amplified from the corresponding gradient fractions and visualized after agarose gel electrophoresis.

identified as *Methylobacillus* sp. Iva (GU937479), while the remaining bands (2, 4 and 5) were unclassified.

Thirty four clones of PCR-amplified 16S rRNA genes were sequenced from the  $^{13}\text{C}$ -enriched DNA fractions. Most of the clones (31 of 34 sequences) affiliated with the genus *Methylobacillus* (Fig. 3). The obtained sequences had less than 95% identity to the other previously described *Methylobacillus* species. Three clone sequences were related to the genus *Stenotrophomonas* within the *Gamma*proteobacteria (Fig. 3) with strong bootstrap support and less than 95% identity to other *Stenotrophomonas* sequences.

#### Identification of triclosan-utilizing bacteria

To verify that members of the genus *Methylobacillus* were utilizing triclosan in the enrichment culture, a specific FISH probe (Meth1138) targeting most members of the genera was designed. The hybridization stringency of the probe was optimized on biomass from the culture and from activated sludge samples and determined to be 25% (v/v) formamide. The probe was used to quantify the relative abundance of *Methylobacillus* in the enriched culture as well as in activated sludge and was calculated to range between 2 and 4% and 0.5 and 1% of the total detected cells, respectively. No further enrichment was detected during SIP or MAR incubations. Dense silver grain patches covering the Meth1138-hybridized cells indicated an active utilization



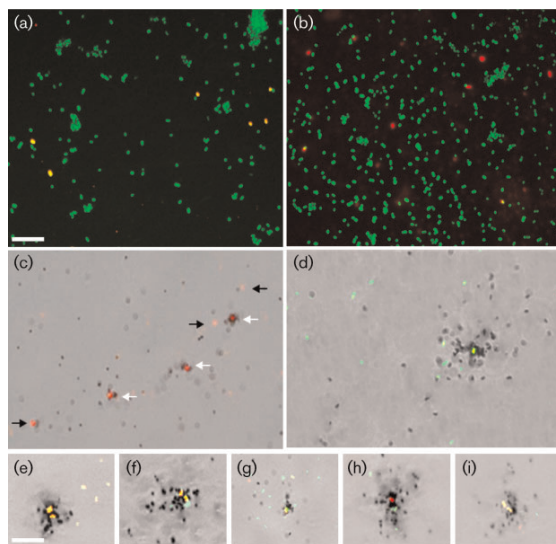
**Fig. 3.** Phylogenetic affiliation of the 16S rRNA gene sequences obtained from the  $^{13}\text{C}$ -enriched SIP fractions. GenBank accession nos or the number of clone sequences obtained are indicated in parentheses. The tree was constructed using the maximum-likelihood algorithm with branching confidence values from 1000 replicates. Bootstrap values  $\geq 75\%$  and  $\geq 90\%$  are indicated by empty and filled circles, respectively. Bar, 5% sequence divergence; the outgroup was made from 10 randomly chosen *Chloroflexi* gene sequences.

of  $^{14}\text{C}$ -labelled triclosan by *Methylobacillus* (Fig. 4). A low background in the MAR visualizations and lack of MAR-positive cells in the pasteurized control indicated a low absorbance and chemography of  $^{14}\text{C}$ -labelled triclosan to the sample. In the enrichment culture, approximately 25% of the Meth1138-positive cells were MAR-positive, but other betaproteobacterial cells (positive with the BET42a probe) were also MAR-positive (Fig. 4). These cells were found to constitute 2–3% of the total number of cells detected by EUBmix and gave similar silver grain density to the MAR-positive *Methylobacillus*.

The oligonucleotide probe was applied to assess the abundance of FISH-detectable *Methylobacillus* bacteria in seven Danish full-scale wastewater treatment plants. With a detection limit of 0.25% of the biovolume, estimated by the use of nonsense probe NONEUB (Wallner *et al.*, 1993), the survey revealed a highly variable presence of bacteria affiliated with *Methylobacillus*; some plants showed a complete absence or around the limit of quantification ( $P < 0.1$ ; Aalborg East, Egå, Hjørring, Skive WWTPs) while others showed relatively high abundance (0.5–2%,  $P < 0.05$ , Bjergmarken, Ejby, Aalborg West WWTPs). The probe hybridized with small, rod-shaped cells (Fig. 4) that

had similar morphology in samples from the enrichment culture and all the activated sludge WWTPs (Fig. 4).

MAR-FISH was also attempted with biomass from a full-scale plant to confirm that these organisms are involved in triclosan removal in these systems. However, due to the presence of very few MAR-positive cells (enumerated to be around 2% of the total number of cells detected by EUBmix, corresponding to approximately  $8 \times 10^6$  cells  $\text{ml}^{-1}$ ) combined with low fluorescence intensities we were not able to assess with confidence the MAR-FISH signals. Design of specific FISH probes targeting the three *Stenotrophomonas* sequences identified by SIP failed to detect target cells and previously published probes for this genus had one mismatch to the sequences obtained from the clone library. However, due to the relatively low abundance of *Gammaproteobacteria* in the enrichment culture (Fig. 4) [ $< 1\%$  positive with the GAM42a probe compared with  $\sim 95\%$  of *Betaproteobacteria* (BET42a) relative to the total FISH positive cells detected by EUBmix] and the observation that all MAR-positive cells were also *Betaproteobacteria*-positive (Fig. 4), no further attempts were taken to verify if members of the *Stenotrophomonas* were taking up  $^{14}\text{C}$ -labelled triclosan.



**Fig. 4.** FISH and microautoradiography images of triclosan-utilizing cells. FISH images of the triclosan-fed enrichment culture after hybridization with (a) the universal bacterial probe EUBmix (green) and probe Meth1138 (red), and (b) probe BET42A (green) and probe GAM42a (red). Cells appearing yellow hybridized with both probes. Representative MAR-FISH images of triclosan-utilizing bacteria present in the enrichment culture incubated for 1 day at 1 mg  $^{14}\text{C}$ -labelled triclosan  $\text{l}^{-1}$  and hybridized with BET42A probe (red) (c, e) or Meth1138 (red) and EUBmix probe (green) (d, f–i). Silver grains surrounding bacterial cells indicate active cellular incorporation of the  $^{14}\text{C}$ -labelled triclosan (white arrows), while black arrows indicate MAR-negative cells. Bars, 10  $\mu\text{m}$ .

## DISCUSSION

Although more than 60 % of total removal of triclosan is attributed to the biodegradation processes in activated sludge treatment (Bester 2003, 2005) very little is known about the micro-organisms involved. Previous studies have shown the ability of a few isolates to degrade triclosan (Gangadharan Puthiya Veetil *et al.*, 2012; Hay *et al.*, 2001; Kim *et al.*, 2011; Meade *et al.*, 2001). However, as these studies rely on the use of culture-dependent methods and do not necessarily reflect the identity of the active members involved in the biodegradation of triclosan *in situ*, the focus of this study was to apply SIP to identify bacteria capable of utilizing triclosan in an enrichment culture. Attempts to apply the SIP approach directly on activated sludge were not successful, most likely because of the low numbers of bacteria involved in the degradation of triclosan as observed in the MAR-FISH results. Apparently, with the amount of  $^{13}\text{C}$ -labelled triclosan added and the sequencing approach used, we were unable to reach sufficient density shift for the labelled DNA during SIP. So, in order to identify triclosan degraders, an enrichment step was introduced. This approach is biased to enrich for triclosan degraders with a low affinity for the substrate, and discriminates against cells with a high substrate affinity.

The enrichment culture, originally started from activated sludge, was fed on regular additions of triclosan and was able to degrade 2 mg triclosan  $\text{l}^{-1}$  with a half-life of 16 h compared with 90 h in the original activated sludge sample. The relatively stable and high removal rate of

triclosan and consecutive development of  $^{14}\text{C}$ -labelled  $\text{CO}_2$  combined with a lack of lag phase in the degradation experiments suggest that the enriched bacterial community has readily adapted to triclosan as a carbon source. The consumption of  $^{13}\text{C}$ -labelled triclosan resulted in a sufficient amount of heavy-labelled DNA, and a shift in the average density of total DNA compared with the unlabelled ( $^{12}\text{C}$ ) control. Generally, to ensure sufficient DNA labelling in SIP experiments, a few doubling times with the labelled substrate is required. This potentially raises concern regarding cross-feeding of labelled carbon. However, we applied a relatively short incubation period and low concentration of the applied  $^{13}\text{C}$ -labelled triclosan to minimize the risk of cross-feeding. The predominant enrichment of a single bacterial clade, the lack of by-products identified, and the confirmation by MAR-FISH with reduced incubation time and tracer, support that the identified *Methylobacillus* are the primary consumers of triclosan in the enrichment culture. The methodological approach of applying SIP with MAR-FISH is a powerful combination that validates the SIP findings and ensures correct interpretation of even organisms with low abundance. The MAR approach requires less uptake of tracer compared with SIP, and is therefore more sensitive and can be used to test uptake of substrate in natural systems under *in situ* conditions. However, we were not able to conclusively verify that *Methylobacillus* was the main triclosan consumer using *in situ* concentrations in the indigenous activated sludge sample due to very low numbers of MAR-positive triclosan degraders.

The finding of a few *Stenotrophomonas*-related clones in the  $^{13}\text{C}$ -labelled DNA clone library could indicate a broader diversity of triclosan degraders or the presence of multiple degradation steps catalysed by different micro-organisms. Although members of *Stenotrophomonas* have previously been shown to be involved in the degradation of environmental pollutants with aromatic structures such as *p*-nitrophenol (Liu *et al.*, 2007), nonylphenol (Soares *et al.*, 2003) and benzene (Lee *et al.*, 2002), their involvement in degradation of triclosan was not confirmed by the MAR-FISH approach. Another betaproteobacterial group was found to be present in similar numbers to *Methylobacillus* and with similar triclosan degradation activity; however, these cells were not identified by the SIP approach. This could be due to insufficient density shift in the SIP fractionation.

Other studies have shown that less than 1 % of the triclosan added to activated sludge is actually transformed into triclosan-methyl, and that the increase of triclosan-methyl corresponded to the decrease of the parent compound (Chen *et al.*, 2011). We attempted to find and identify triclosan degradation by-products from the enrichment culture by GC-MS and revealed the presence of 2,4-dichlorophenol but this was below the limit of quantification. The lack of accumulated by-products and development of labelled  $\text{CO}_2$  in the head space during incubation with  $^{14}\text{C}$ -triclosan indicates that the added triclosan was fully mineralized. Alternatively, the findings could suggest the presence of a more metabolically diverse community of triclosan degraders in activated sludge, but these would typically be present in small numbers and therefore difficult to identify.

*Methylobacillus* belongs to methylotrophs, which is a phenotypically defined group capable of using one-carbon compounds as the sole source of energy and carbon (Hanson & Hanson, 1996). However, it has been shown that several methylotrophs that are within the genus *Methylobacillus* can degrade organic compounds through co-metabolism, such as the pesticide carbonfuran and choline (Hanson & Hanson, 1996), or through direct metabolism, such as microcystin (Hu *et al.*, 2009). Other methylotrophs are known for their ability to participate in the co-metabolic degradation of various environmental pollutants, including trichloroethylene, phenol and different aromatic compounds (Chongcharoen *et al.*, 2005; Koh *et al.*, 1993; Tsuji *et al.*, 1990). Metabolic pathway analyses have shown that *Methylobacillus* contains unique clusters of genes encoding the degradation of chlorocatechol, a major intermediate product in the biodegradation of chloroaromatic compounds (Caspi *et al.*, 2012; Spokes & Walker, 1974). Little information is available regarding the biodegradation products of triclosan, although catechol and 3,5-dichlorocatechol were detected when triclosan was degraded by pure cultures of *Pseudomonas*-like strains (Gangadharan Puthiya Veetil *et al.*, 2012) and *Sphingomonas* sp. PH-07 incubated with diphenyl ether (Kim *et al.*, 2011). This information supports the notion that members of the genus *Methylobacillus* may play a role in triclosan degradation

in the enriched culture and in WWTPs. To our knowledge, organisms within this group have not previously been linked to triclosan degradation. The FISH surveys in the seven Danish WWTPs show that *Methylobacillus* are indeed present in activated sludge although they are more abundant than can be ascribed to degradation of micro-pollutants such as triclosan, and the abundance thus indicates that they are involved not only in degrading aromatic micro-pollutants but likely also in other processes as well.

In conclusion, SIP combined with MAR-FISH was used here to identify the active community responsible for the degradation of triclosan within an enrichment culture originating from activated sludge. The findings show the ability of members of the genus *Methylobacillus* to utilize triclosan. Identifying the specific organisms involved in triclosan degradation provides valuable information that may lead to possible strategies to enhance micro-pollutant removal.

## ACKNOWLEDGEMENTS

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### **Research paper 8:**

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# **BIODEGRADATION OF TRICLOSAN AND FORMATION OF METABOLITES IN ACTIVATED SLUDGE UNDER AEROBIC CONDITIONS**

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## **Abstract**

Aerobic degradation experiments of Triclosan were performed in activated sludge to identify possible degradation pathways for this compound. An almost complete removal of Triclosan was achieved within 7 days, with formation of biodegradation products such as 2,4-dichlorophenol, 4-chlorocatechol, Monohydroxy-Triclosan and Dihydroxy-Triclosan in the liquid phase and a hitherto unknown transformation product Triclosan-O-Sulfate was identified in the sludge. Based on the analysis of biodegradation products, three main lines of reactions are identified: 1) chemical scission of ether bond to form phenols and catechols, 2) introduction of OH group to the aromatic ring, and 3) adding methyl or sulfate groups to the original hydroxyl group. The half life of the metabolites indicate that they may be emitted from waste water treatment plants.

## Introduction

Triclosan (2,4,4'-trichloro-2'-hydroxydiphenyl ether, CAS: 3380-34-5) was first introduced in the health care industry in 1972 and later as an additive to toothpaste in Europe (Jones et al., 2000). Due to the bactericidal property and the chemical stability, Triclosan became popular as an antimicrobial substance in a wide range of applications and the demand of it has been growing rapidly in last 40 years (Fiss et al. 2007). The consumption within the EC reached approximately 450 tons (as 100% active) in 2006 (SCCP/1192/08, 2009).

Triclosan can inhibit bacteria by blocking lipid biosynthesis, which is necessary for building cell membranes components (Schweizer, 2001). Considering the toxicological profile and its widespread detection in the surrounding environment, there is a growing concern about the potential impact of Triclosan on human and ecosystem health, such as skin irritation, allergy susceptibility, and also eco-toxicity to the aquatic and terrestrial environment (Wilson et al., 2003; Coogan et al., 2007; Franz et al., 2008). Therefore, there is an increasing attention on the fate of Triclosan in the environment.

The widespread use of Triclosan and incomplete removal in wastewater treatment plants (WWTPs) indicates that Triclosan may enter the environment via the sludge or via WWTPs effluent. Previous studies described Triclosan as one of the most commonly encountered substances in aqueous and terrestrial ecosystems (Lindström et al., 2002; Ying and Kookana, 2007; Kumar et al., 2010). In addition, it has been detected in fish, soil, and sediments as it has high bioaccumulation properties (Coogan et al., 2007; Xie et al., 2008; Lozano et al., 2010; Fernandes et al. 2011).

As Triclosan is used widely and disposed mostly “down-to-the-drain”, it has been detected in numerous municipal wastewater influent samples at concentrations of several µg/L (Bester, 2005; Wu et al., 2007; Kumar et al., 2010; Nakada et al., 2010). In conventional activated sludge WWTPs about 90% removal of Triclosan was detected (Singer et al. 2002; Bester 2003; 2005). In fact, both biodegradation and physical/chemical sorption to activated sludge may explain the removal fate of Triclosan in WWTPs. About 30-50% of the Triclosan can be recovered from sludge as Triclosan is relatively lipophilic with log  $K_{ow}$  of 4.2-4.8 and log  $K_{oc}$  of 4.3 (Singer et al., 2002; Bester, 2003, 2005; Coogan et al., 2007; Heidler and Halden, 2007; Ying et al. 2007). On the other hand, only about 5% is detected in the discharge of the WWTPs (Singer et al., 2002, Bester, 2003, 2005), which indicate that the majority of the removal is due to biodegradation processes (Singer et al., 2002; Bester, 2003; Heidler and Halden, 2007). In sludge biodegradation Triclosan-Methyl has been detected and is contributing up to 5% of the mass balance (McAvoy et al., 2002; Lindström et al., 2002; Bester, 2003, 2005). Previous research (Chen et al., 2011) demonstrated this methylation to occur under aerobic conditions. Additionally, 2,4-dichlorophenol is a well known degradation product of Triclosan which has been detected by several investigators within sludge biodegradation experiments (Kim et al., 2010, Lee et al., 2012). Chlorocatechol, Monohydroxy-Triclosan and Dihydroxy-Triclosan have also been proposed as biodegradation products of Triclosan by bacteria (Kim et al., 2010; Veetil et al., 2010; Lee et al., 2012), however structural confirmation by true standards or NMR was not achieved in those studies. Triclosan-O-Sulfate has been described as transformation

product of Triclosan in rat liver (Wu et al., 2010) and carrot (Macherius et al., 2012), however confirmation with NMR or true standards are missing.

Triclosan is toxic, lasts for a long time in the environment, and may travel long distances from their sources of usage, release and emission. As some of the intermediate products of Triclosan transformation are missing, studies of chemical and biological conversions of Triclosan are important for risk assessments of ecosystems and human health. The aim of this work is to investigate the biodegradation of Triclosan by activated sludge under aerobic conditions, in order to achieve a better knowledge of the metabolites generated as well as their biodegradation rates.

## **Materials and methods**

### **Activated sludge sampling**

Activated sludge samples were collected from Bjergmarken WWTP (Roskilde, Denmark), which processes  $6 \times 10^6$  m<sup>3</sup> wastewater (125,000 population equivalents, PE) annually. About 80% of the received wastewater is domestic. This WWTP includes primary sedimentation basins, activated sludge treatment basins and a final clarifier before the water is released to the nearby fjord. It operates biological phosphorus removal and alternating nitrification/denitrification, and produces about 1200 tones dry sludge annually. The suspended solids (SS) content of the activated sludge was 4 g L<sup>-1</sup> and its volatile solids content was 2.5 g L<sup>-1</sup> during the sampling period.

### **Degradation experiments**

Biodegradation experiments were carried out in 5 L glass bioreactor with starting concentrations of 5 mg L<sup>-1</sup> Triclosan, which is somewhat higher than found in real WWTPs. The high concentration was chosen to be able to identify the Triclosan metabolites in sludge. During the experiments, the reactor was maintained at 17 ± 2 °C and the pH value remained constant (6.9 ± 0.5) during the experiment. The incubation conditions were maintained under aerobic conditions by supplying air through a diffuser stone with a flow rate of 1.3 L h<sup>-1</sup>. The reactor was stirred by means of teflonized magnetic stir bars to keep the sludge homogeneous. No additional carbon source was added to the system, thus it was run as static reactors. Sludge samples were taken every day from the reactor. Detailed experiment procedures were described previously (Chen et al., 2011).

#### **Extraction and instrumental analysis**

##### **Direct injection of aqueous samples**

To determine the concentration of Triclosan and possible metabolites, 1 mL sludge samples were taken from the reactor and centrifuged (10,000 x *g* for 10 min at room temperature) by Microcentrifuge 157 (Ole Dich Instrumentmakers, Hvidovre, Denmark) to remove the particles and the supernatants were injected directly into HPLC-MS/MS.

##### **Solid phase extraction**

To obtain better signals of metabolites, 30 mL sludge samples were taken every day from the reactor. Samples were centrifuged (10,000 x *g* for 10 min at room temperature) to separate the liquid and solid phase. The liquid phase (supernatant from centrifuge) were extracted by means of solid phase extraction (SPE) with SDB2 column (Mallinckrodt Baker, Deventer, The Netherlands) with 12 mL methanol, whereas the solid



phase (pellets from centrifuge) were re-extracted by methanol and centrifuged to remove the particles. All methanolic extracts were then concentrated to 1 mL with a nitrogen flow condenser at 55 °C.

#### Instrumental analysis

The samples were analyzed by liquid chromatography with tandem mass spectrometric detection (HPLC-MS/MS). The separation was performed using a Phenomenex synergis 4u polar-RP column (150×2 mm I.D., particle size 4 µm). The flow rate was 0.25 mL min<sup>-1</sup>. Considering the characteristics of electrospray ionization and the chemical properties of Triclosan and potential metabolites, preliminary experiments were performed to test which conditions (neutral, acidic, alkaline) provide the optimum response for the mobile phase in presence of methanol and water. The results showed that Triclosan and metabolites achieved better separation and ionization efficiency when analyzed in a mobile phase consisting of methanol and water containing 0.2% formic acid. Therefore, the LC gradient was established by mixing two mobile phases: phase A, HPLC water (0.2% formic acid) and phase B, Methanol (0.2% formic acid). The chromatographic separation was achieved with the following gradient: 0-2 min 100% A, changing to 100% B in 11 min, keep constant for 4min with 100% B and return to initial conditions in 0.5min. The injection volume was 100 µL for the water samples and 10 µL for the extracted sludge samples.

The LC system consisted of a Dionex UltiMate 3000 autosampler (WPS-3000T SL), a Dionex UltiMate 3000 pump (HPG-3600 M), a Dionex UltiMate 3000 column compartment hold (TCC-3000 RS) on 20 °C (all from Dionex, Sunnyvale, California, United States). After LC separation, the analytes were determined by an AB-Sciex

(California, United States) API 4000 triple quadrupole mass spectrometer using electrospray ionization in negative mode. Nitrogen was used as a drying (at 400°C) nebulising and collision gas. One scan per second was recorded. The compounds were characterized by scans with the first quadrupole, as well as daughter spectra, while they were quantified using multireaction monitoring (MRM).

High resolution mass spectrum (HRMS/MS) was used for further sample analysis. The detection was performed using a LTQ-Orbitrap-MS (LTQ Orbitrap Velos, Thermo Scientific, Bremen, Germany) by direct injection using a syringe pump (flow rate: 8  $\mu\text{L min}^{-1}$ ) with ESI operated in negative ionization mode.

Similar oxidation products as described for ozonisation of Triclosan (Chen et al., 2012) was examined in this study. For further structure confirmation Triclosan oxidation products were produced by reacting 24 mg of Triclosan with aqueous ozone and about 1 mg of hydroxy- Triclosan (OH-Triclosan) and dihydroxy- Triclosan ((OH)<sub>2</sub>-Triclosan) were isolated as described elsewhere (Chen et al., 2012). Purified Triclosan oxidation products were analysed by Nuclear Magnetic Resonance (NMR). The dried fractions from the HPLC separation were redissolved in 40  $\mu\text{L}$  methanol-d<sub>4</sub> which contained 0.03% (v:v) of tetramethylsilane (TMS). Spectra were recorded at 298.1K on a BRUKER AVIII-600MHz NMR spectrometer equipped with a 1.7mm TXI (H/C/N) probe. <sup>1</sup>H-NMR, <sup>13</sup>C-NMR, [<sup>1</sup>H, <sup>1</sup>H]-2QF-COSY, [<sup>13</sup>C, <sup>1</sup>H]-HSQC and [<sup>13</sup>C, <sup>1</sup>H]-HMBC spectra were recorded.

## **Materials**

For analytical purposes Triclosan, was purchased from Ehrenstorfer (Augsburg, Germany) with purity being  $\geq 99\%$  according to the supplier. For transformation studies

Triclosan (Irgasan) obtained from Sigma-Aldrich (Steinheim, Germany) was used. 2,4-dichlorophenol and 4-chlorocatechol were also purchased from Sigma-Aldrich. Methanol was used in residue grade quality and purchased from Merck, Darmstadt, Germany. Methanol-d<sub>4</sub> containing 0.1% tetramethylsilane (TMS) was purchased from Cambridge Isotope Laboratories, Andover, MA (USA). Triclosan-O-Sulfate was synthesized by Toronto Research Chemicals (Toronto, Canada).

## Results and discussions

Figure 1 shows Triclosan and Triclosan-metabolite concentrations during a degradation experiment of 220 hours. Nearly all Triclosan was degraded within 150 hours, after which the Triclosan concentrations remained almost constant at less than 0.01 mg L<sup>-1</sup> to the end of the experiment (220 h).

While Triclosan was detected at retention time (RT) of 15.88 min, the following metabolites were detected in the aqueous phase:

Monoaromatic compounds at RT 12.79 min and 14.02 min were identified and verified with true standards as 4-chlorocatechol and 2,4-dichlorophenol, respectively.

Monohydroxy-Triclosan (OH-Triclosan). Several isomers were identified indicating presence of an unselective oxidation of the aromatic ring system (Fig.2 and S1). However, the isomer at RT 14.88 min was identified as one that could be synthesized and isolated (S1). Submitting the synthesized oxidation products with the same retention time as the biogenerated one to NMR measurements, revealed the identity of

the main Monohydroxy-Triclosan to be 5-OH-Triclosan (Compare to Chen et al., 2012) (Table 1).

Dihydroxy-Triclosan ((OH)<sub>2</sub>-Triclosan). Several isomers were identified at RT 15.55 min (Fig.2).

Triclosan-O-Sulfate was detected in the extracted pellet of the centrifuged sludge. Fig.3 shows the chromatogram and the spectrum of this compound. The most abundant ions are m/z 367, 369 and 371, corresponding to a molecule containing three chlorine atoms (M-1). To obtain more information of the unknown product, high resolution of mass spectrum was used. S3 (a) shows the mass [M-H]<sup>-</sup> of the unknown compound analysed by HRMS with 366.9002, from which the product ion was detected at 286.9434 shown in S3 (b). After comparison with the theoretical masses, the unknown compound could be identified as Triclosan-O-Sulfate (C<sub>12</sub>H<sub>6</sub>Cl<sub>3</sub>O<sub>5</sub>S), which has theoretical mass at m/z of 366.8996. The product ion could be confirmed to be Triclosan with theoretical mass of 286.9434 after a cleavage of O<sub>3</sub>S (theoretical mass 79.9563). For further confirmation, Triclosan-O-Sulfate was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada), which produced a peak with the same retention time and mass spectrum as the metabolite detected in the sample.

Figure 4 shows the proposed degradation products resulting from the biodegradation of Triclosan in activated sludge systems. The breaking of the ether bond seems to be the preferred pathway, thus the major degradation product obtained after the degradation of Triclosan was the compounds yielding an [M-H]<sup>-</sup> ion at 161, which shows the maximum intensity in the chromatogram. Following the same degradation pathway, other degradation product at m/z 143 could also be detected.

Another degradation pathway observed was the substitution of the aromatic hydrogen atom(s) by hydroxyl group(s), leading to the structures proposed for m/z 303 and 319. Similarly, the degradation product Triclosan-Methyl could be the result of the replacement of the hydrogen atom by methyl group, which has been described elsewhere (Chen et al., 2011). Furthermore, the replacement of the hydrogen group by sulfate has been detected leading to the formation of Triclosan-O-Sulfate.

Quantitative analysis was also carried out to quantify the metabolites. As shown in Figure 1, the concentration of 2,4-dichlorophenol started from 50 ng/mL, reached 320 ng/mL after 48 hours, and then decreased to about 50 ng/mL. The increase in concentration of 2,4-dichlorophenol indicated the biotransformation of 2,4-dichlorophenol from Triclosan, the decrease concentration after 48 hours suggested that 2,4-dichlorophenol is not the final product and it will be further degraded. It could be assumed that about 7% of Triclosan was transformed into 2,4-dichlorophenol in the reactors within 50 hours. Similarly, concentration of Triclosan-O-Sulfate increased from 0 to 256 ng/mL, corresponding to 7% of transformation from Triclosan within 120 hours. The concentration of 4-chlorocatechol could not be quantified as the signal was below the limit of quantification. Concentrations of Monohydroxy-Triclosan and Dihydroxy-Triclosan, could not be determined as the standards are not commercially available. However, the peak area of those compounds showed the same tendency as 2,4-dichlorophenol as shown in Figure 7, which suggested that they are originated from Triclosan and they are intermediate products which are also biodegradable in activated sludge system.

## **Conclusion**

Considering the biodegradation products, it was tentatively concluded that for relatively high concentrations of Triclosan, direct biodegradation consisted of at least four parts: 1) the C-O bond cleavage producing 2,4-dichlorophenol and 4-chlorocatechol. 2) substitution of the aromatic hydrogen atom(s) by hydroxyl group(s) producing OH-Triclosan and (OH)<sub>2</sub>-Triclosan. 3) replacement of the 2-OH-hydrogen atom by methyl group forming Triclosan-methyl. 4) replacement of the hydrogen atom by sulfate group leading to the formation of Triclosan-O-Sulfate.

At the experiment conditions, 1% of Triclosan has been transformed into Triclosan-methyl, 7% has been transformed into 2,4-dichlorophenol and 7% has been transformed into Triclosan-O-Sulfate. All the transformation products detected in the experiment were intermediate products which are also biodegradable in activated sludge system. However, considering the kinetics obtained in these experiments it must be concluded these compounds are most probably emitted via the WWTP effluents into surface waters as WWTPs usually operate with hydraulic residence times of about 20 hours.

## **Acknowledgement**

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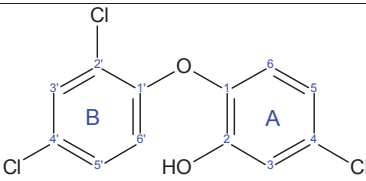
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Chen, X.J.; Richard, J.; Liu, Y.L.; Dopp, E.; Türk, J.; Bester, K. Ozonation products of triclosan in advanced wastewater treatment. *Water Res.* **2012**, 46, 2247-2256.

## Tables and Figures

Table 1. Triclosan structure and chemical shifts ( $\delta$  [ppm], at 298.1K in methanol-d<sub>4</sub>, referenced against internal TMS) detected in NMR analysis.

Triclosan structure		Triclosan		5-OH-Triclosan	
	Atom	H	C	H	C
	1	-	142.58	-	142.74*
	2	-	150.13	-	142.11*
	3	6.95	117.51	6.93	118.11
	4	-	130.48	-	116.46
	5	6.82	120.06	-	146.63
	6	6.82	121.69	6.46	108.74
	1'	-	152.79	-	152.63
	2'	-	125.04	-	125.05
	3'	7.49	130.48	7.49	130.19
	4'	-	128.29	-	128.33
	5'	7.21	128.24	7.23	128.26
	6'	6.76	119.05	6.82	119.38

\* ambiguous assignment (position 1 and 2 cannot be clearly assigned separately)

Figure 1. Concentration of Triclosan and formation of the metabolites 2,4-dichlorophenol and Triclosan-O-Sulfate in reactor during sampling period. Concentration of Triclosan and 2,4-dichlorophenol were measured in the aqueous phase, whereas concentration of Triclosan-O-Sulfate was measured in sludge phase. Triclosan concentrations refer to the left y-axis, the transformation products are displayed with reference to the right y-axis.

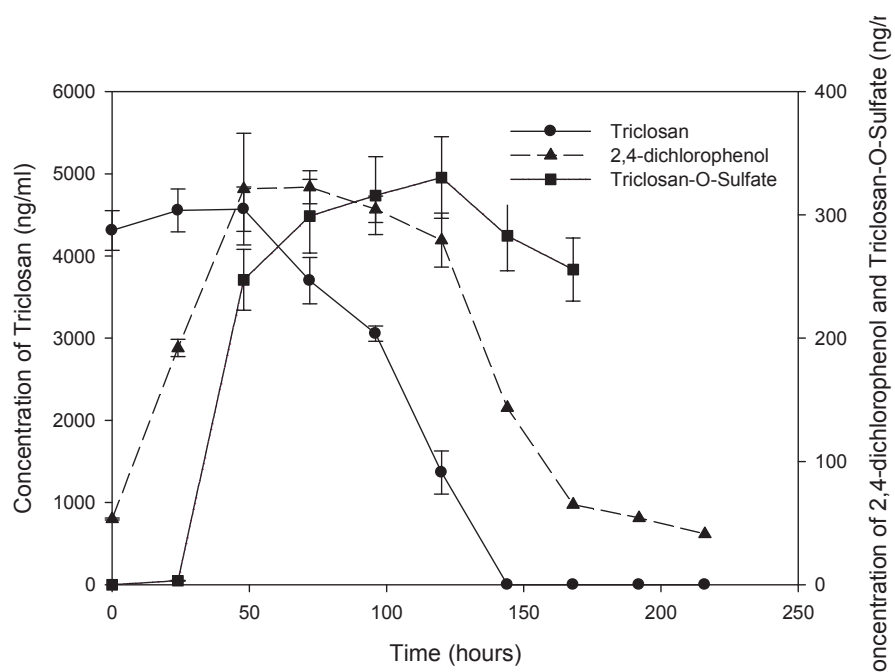


Figure 2. Chromatogram of a sludge incubation (liquid phase) detected by means of HPLC-MS/MS with transformation products identified.

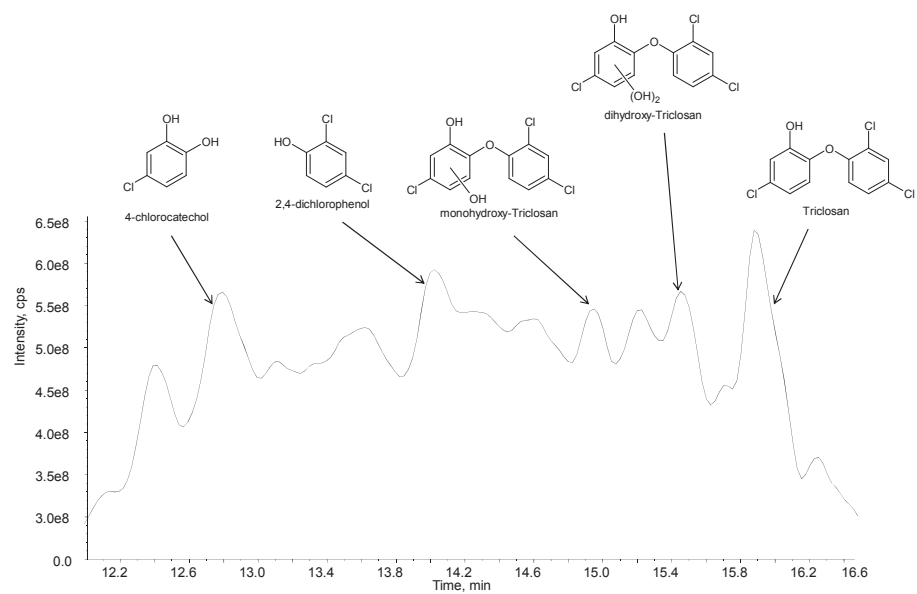


Figure 3. HPLC-MS/MS analysis of the transformation product identified in the solid phase. The full chromatogram of the sample (a) and the background-subtracted mass spectrum of Triclosan-O-Sulfate (b).

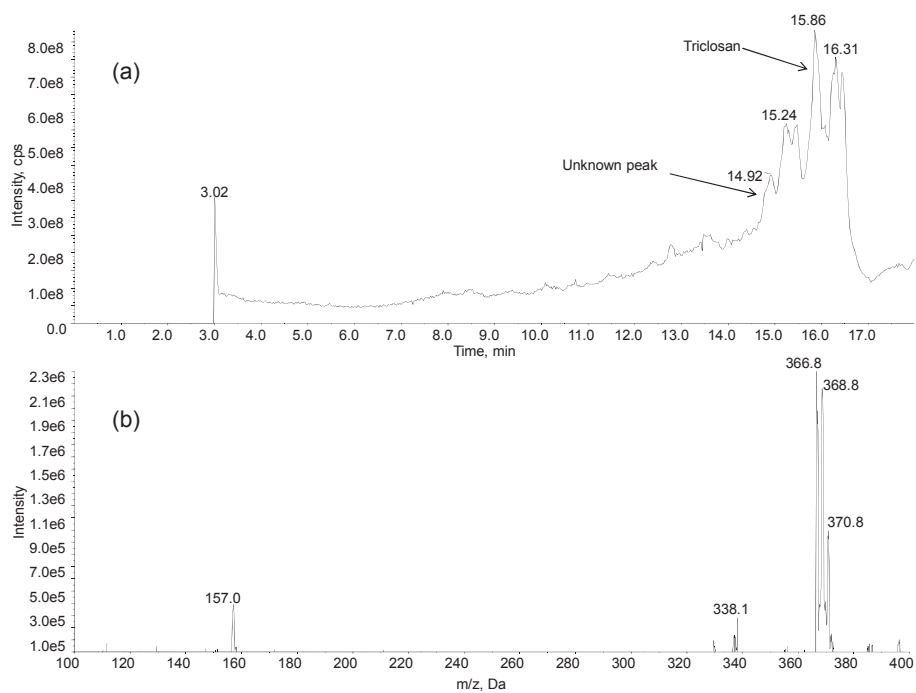
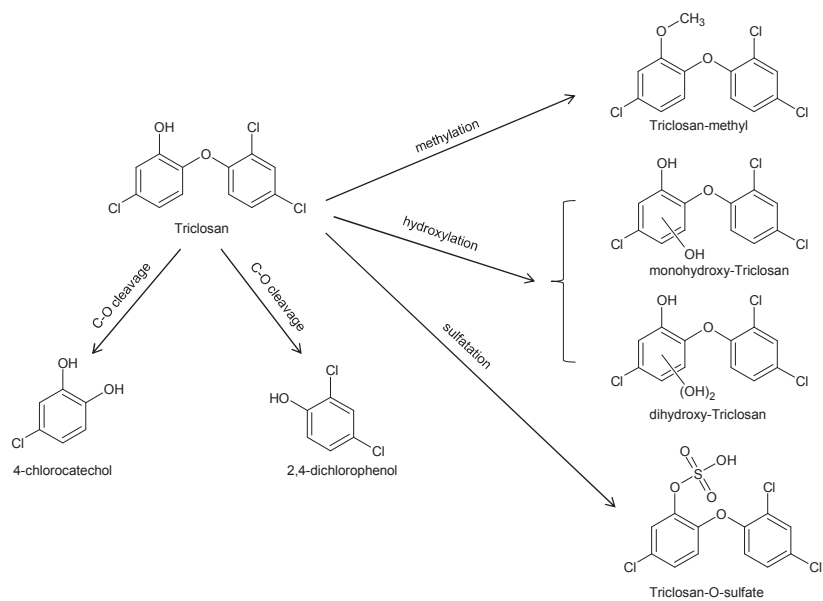


Figure 4. Proposed biodegradation pathway of Triclosan.



**Supplementary Material for**

**BIODEGRADATION OF TRICLOSAN AND FORMATION**

**METABOLITES IN ACTIVATED SLUDGE UNDER**

**AEROBIC CONDITIONS**

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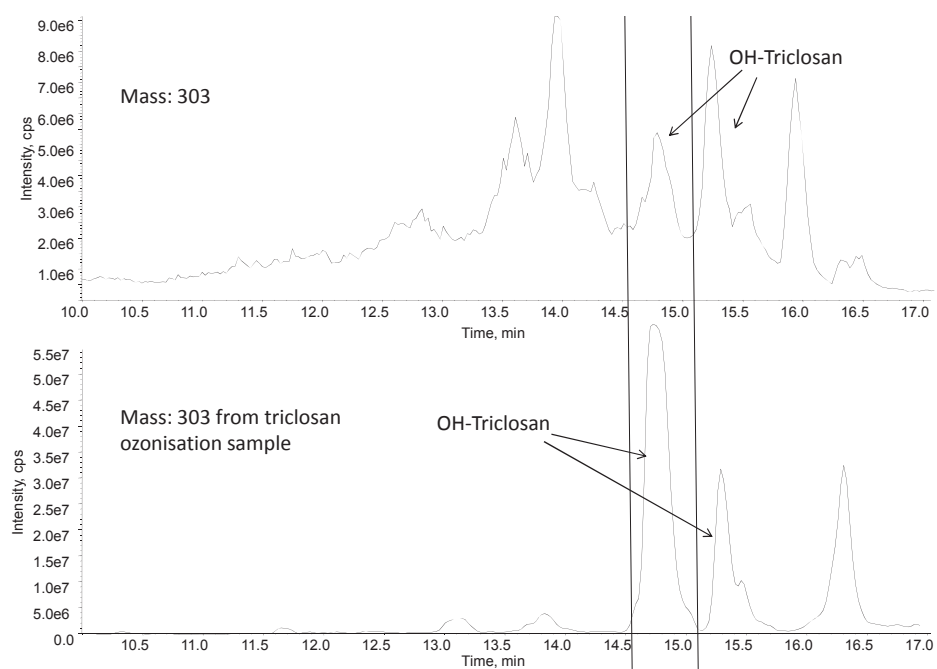
2) Federal Institute of Hydrology (BfG), Koblenz, Germany

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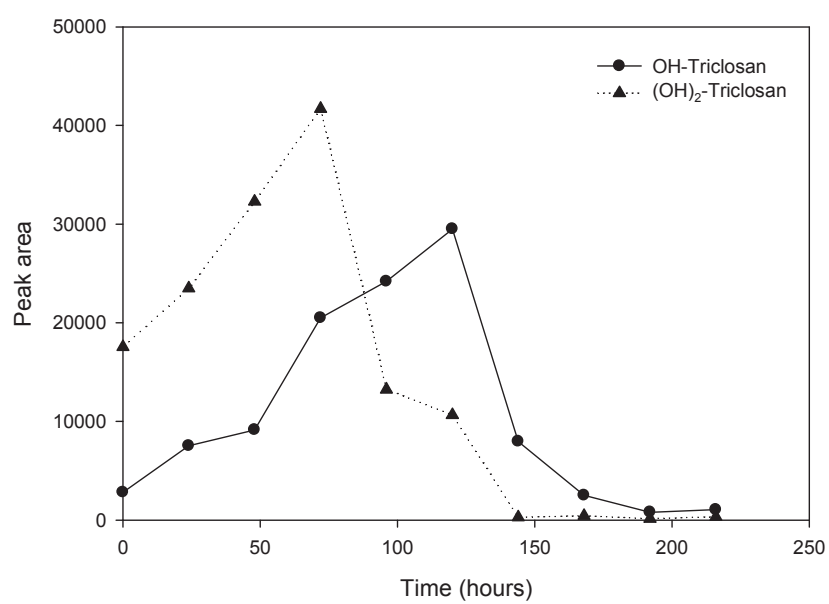




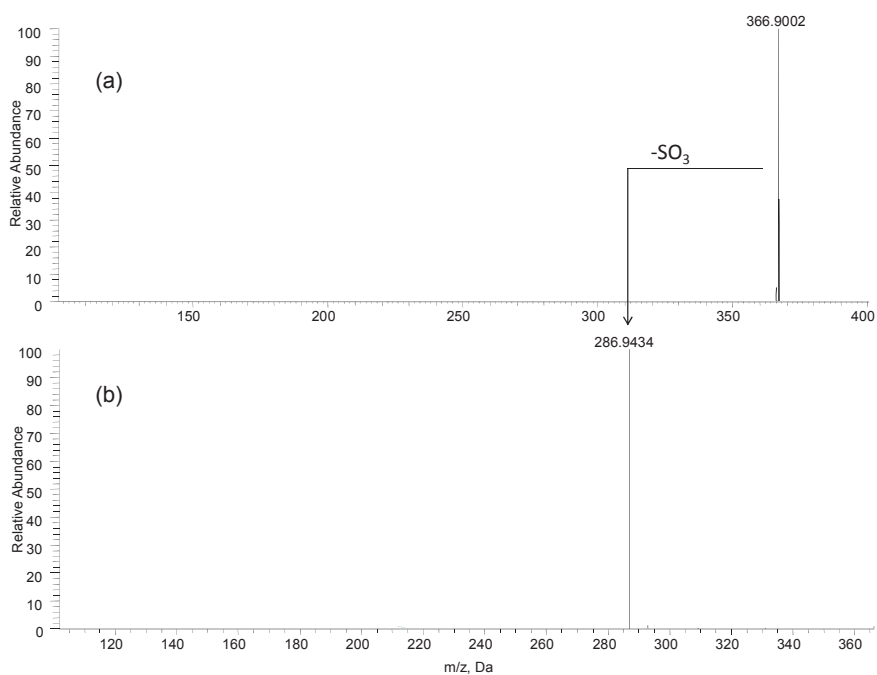
Supplementary material S1. Pattern of different monohydroxy-Triclosan derivatives from incubation with sludge in comparison with sample from Triclosan ozonisation (Chen et al., 2012).



Supplementary material S2. Development of relative concentrations of monohydroxy- and dihydroxy-Triclosan during the experiment period.



Supplementary material S3. HRMS/MS analysis of the Triclosan-O-Sulfate in the solid phase. High resolution mass spectrum of the unknown peak (a) and the daughter mass spectrum of mass 366.9002 (b), triclosan (mass 286.9434) was formed after a cleavage of 79.95676 ( $\text{O}_3\text{S}$ ).



## Mandatory page

Thesis title: Triclosan removal in wastewater treatment processes

Name of PhD student: Xijuan Chen

Supervisors: Jeppe Lund Nielsen, Kai Bester

List of published papers:

- Paper 1: **Chen XJ.**, Pauly U., Rehfus S. and Bester K. Personal care compounds in a reed bed sludge treatment system, Chemosphere 76 (2009) 1094–1101
- Paper 2: **Chen XJ.**, Pauly U., Rehfus S. and Bester K. Removal of personal care compounds from sewage sludge in reed bed container (lysimeter) studies — Effects of macrophytes, Science of the Total Environment 407 (2009) 5743–5749
- Paper 3: **Chen XJ.** and Bester K. Determination of organic micro-pollutants such as personal care products, plasticizer and flame retardants in sludge, Anal Bioanal Chem (2009) 395:1877–1884
- Paper 4: Bester K., **Chen XJ.**, Pauly U. and Rehfus S. Abbau von organischen Schadstoffen bei der Klärschlammbehandlung in Pflanzenbeeten, Korrespondenz Abwasser, Abfall 58 (2011) 1050-1157
- Paper 5: **Chen XJ.**, Nielsen JL., Furgal K., Liu YL., Lolas IB. and Bester K. Elimination of triclosan and formation of methyl-triclosan in activated sludge under aerobic conditions, Chemosphere 84 (2011) 452–456
- Paper 6: **Chen XJ.**, Richard J., Dopp E., Türk J., Liu YL. and Bester K.

Ozonisation products of triclosan in advanced wastewater treatment, Water Research 46 (2012) 2247-2256

- Paper 7: Lolås IB., **Chen XJ.**, Bester K. and Nielsen JL. Identification of triclosan degrading bacteria using stable isotope probing and microautoradiography. Microbiology 158 (2012) 2805-2814
- Paper 8: **Chen XJ.**, Wimmer R., Ternes T., Schlüsener M., Nielsen JL. and Bester K. Biodegradation of triclosan and Formation metabolites in activated sludge under aerobic conditions. Manuscript in preparation

This thesis has been submitted for assessment in partial fulfillment of the PhD degree. The thesis is based on the submitted and published scientific papers which are listed above. Parts of the papers are used directly or indirectly in the extended summary of the thesis. As part of the assessment, co-author statements have been made available to the assessment committee and are also available at the Faculty of Engineering and Science, Aalborg University.

